Species interactions in a grassland mixture under low nitrogen fertilization and two cutting frequencies. II. Nutritional quality

Ergon Å.[1*], Kirwan L.[2], Fystro G.[3], Bleken M.A.[4], Collins R.P.[5], Rognli O.A.[1]

[1]Norwegian University of Life Sciences, Department of Plant Sciences, P.O. Box 5003, N-1432 Ås, Norway

[2]UCD Institute of Food and Health, Science Centre South, University College Dublin, Belfield, Dublin 4, Ireland

[3]Norwegian Institute of Bioeconomy Research, P.O. Box 115, N-1431 Ås, Norway

[4]Norwegian University of Life Sciences, Department of Environmental Sciences, P.O. Box 5003, N-1432 Ås, Norway

[5]Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Gogerddan, Aberystwyth, UK

Correspondence to: Åshild Ergon, Norwegian University of Life Sciences, Department of Plant Sciences, P.O. Box 5003, N-1432 Ås, Norway

E-mail: ashild.ergon@nmbu.no

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Abstract

Mixtures and pure stands of perennial ryegrass, tall fescue, white clover and red clover were grown in a 3-cut and a 5-cut system in southern Norway, at a low fertilization rate (100 kg N ha^{-1} $year^{-1}$). The nutritional quality (annual weighted averages) of the dried forage from the two first harvesting years was analysed. There was no significant effect of species diversity on crude protein (CP) concentration. In the 3-cut system we found a significant species diversity effect leading to 10% higher ADF (acid detergent fibre) concentrations, 20-22% lower WSC (water soluble carbohydrate) concentrations and 4% lower NE_L (net energy for lactation) concentrations in mixtures as compared to pure stands (averaged across the two first years). In the 5-cut system similar effects were seen in the first year only. This diversity effect was associated with a reduction in WSC and NE_L concentrations and an increase in ADF, NDF and CP concentrations in the grass species, and not in red clover, when grown in mixtures. This is thought to be a combined result of better N availability and more shading in mixtures. Species diversity reduced the intra-annual variability in nutritional quality in both cutting systems.

Keywords

Festuca arundinacea, forage quality, *Lolium perenne*, species diversity, *Trifolium pratense, Trifolium repens*

Introduction

There are marked differences in the nutritional composition of grasses and legumes, with legumes having a higher concentration of crude protein (CP) and a lower concentration of fibre than grasses (e.g. Hatfield *et al.,* 2007). The digestibility of all forages is reduced as a consequence of reproductive development due to an increase in the proportion of stems and lignified cellulose, accompanied with an increase in fibre concentration and a decrease in CP and readily digestible carbohydrates. The rate of decrease in digestibility with reproductive development tends to be lower in legumes than in grasses (Moore and Jung, 2001). There are also differences among species of legumes and grasses, which are partly a result of variations in growth habit and the allocation of resources between stem and leaf tissues. The timing of reproductive development and stem elongation varies among and within species, as does the tendency to produce stems during regrowth after defoliation. As a result of the strong effect of heading, stem development, and the age of tissues (Duru, 2008), nutritional composition is highly influenced by the defoliation regime, with earlier and more frequent defoliation resulting in forage of higher digestibility (Gardarin *et al*., 2014).

The inclusion of legumes in grassland swards has several advantages, such as providing N to the grasses through symbiotic N fixation, contributing to the dry matter (DM) overyielding which is often obtained in species mixtures, and increasing the voluntary intake of forage by livestock due to attributes that increase the rate of passage through the rumen (reviewed by Lüscher *et al.,* 2014). Nutritional quality is also more stable across harvests in grass-legume mixtures than in pure stands of grasses (Sleugh *et al.,* 2000; Sanderson, 2010). This is partly because grasses are generally earlier in reproductive development than legumes, resulting in the effects of reproductive development being distributed over a larger time span and balanced by the presence of species at other developmental stages. The nutritional quality of mixtures is largely determined by the dominant species in the mixture, and therefore it may change as the species composition changes from year to year (Deak *et al.,* 2007; Sturludottir *et al.,* 2013; Brink *et al.,* 2015). There are few reported examples of species diversity effects independent of sampling effects occurring among the species in a sward. Species diversity may also improve nutritional quality through the repression of weeds (Tracy *et al.,* 2004; Picasso *et al.,* 2008) if these have lower nutritional value. There is limited information available on how the nutritional composition of forage plants is affected by plant interactions through N fixation or competition for light, water and nutrients. We conducted an experiment with a four-species mixture sown with variable species proportions and cultivated at a low N-input level and in two different cutting systems. The effects of species diversity and cutting frequency on DM yield and changes in botanical composition over time in the same experiment were described by Ergon *et al.* (2016). Here, we analysed the effects of species diversity on the swards' nutritional quality and intra-annual stability over the two first harvesting years. We asked the following questions:

1) Are there species diversity effects on forage nutritional variables?
2) Are any such diversity effects affected by cutting frequency?
3) What effect does species diversity have on intra-annual stability of nutritional quality?

Materials and methods

Field experiment

A field experiment, described in detail in Ergon *et al*. (2016), was conducted at Ås, Norway. In brief, pure stands and mixtures of perennial ryegrass (*Lolium perenne* L.), tall fescue (*Festuca arundinacea* Schreb.), red clover (*Trifolium pratense* L.) and white clover (*T. repens* L.) were sown in 2010 in a split plot design with cutting system (3 or 5 cuts per year) as main plots, and sward type as subplots. Fertilizer was applied at a rate equal to 100 kg N ha^{-1} $year^{-1}$. After each harvest in 2011 and 2012, the harvested material within each plot was mixed, and a sample (approximately 1 kg) was taken from all plots of one of the two seed-rate treatments (20 kg ha^{-1}) in the experiment. In order to study the effect of sward type on nutritional variables of single species, samples sorted into species fractions were also taken at the first and last harvest in the 3-cut system in 2012.

Analysis of nutritional quality

Samples were dried at 60 ^{o}C and cut into smaller pieces. Representative subsamples were milled in a Cyclotec 1093 sample mill (Foss A/S, Hillerød, Denmark) with a 1 mm sieve, and scanned with an NIR spectrophotometer (NIRSystems 6500, Silver Spring MD, USA). The content of crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), non-fibre carbohydrates (NFC), water-soluble carbohydrates (WSC), net energy for lactation (NE_L), digestible energy (DE) and metabolizable energy

(ME) were determined in approximately 20% of the samples by chemical analyses of CP, NDF, ADF, NFC and WSC and calculations based on these variables, at Dairy One Forage testing laboratory, Itacha, NY USA. Energy variables were estimated according to Weiss *et al.* (1992), Van Soest and Fox (1992), Weiss (1993) and Weiss (1995). NIR data analysis, including collection of spectra, selection of samples for chemical analysis, local calibration and prediction, was conducted using ISI software (NIRS2, ver. 4.00, Intrasoft International, Silver Spring MD, USA). Calibration and validation statistics are shown in a supplementary file (Table S1) as Supporting Information in the online version of this paper.

Statistical analysis

For each of the nutritional components the weighted average concentration across harvests,

$$\sum_{i=1}^{h}\left(concentration_i \times \frac{dry\ matter\ yield_i}{dry\ matter\ yield_{total}}\right),$$

and the annual yield of the nutritional component,

$$\sum_{i=1}^{h}\left(concentration_i \times dry\ matter\ yield_i\right),$$

where i is the harvest number and h is the total number of harvests per year, were calculated for each plot and year. The average values for each year and treatment are shown in a supplementary file (Table S2) as Supporting Information. The effect of cutting system, the species identity effects $\beta_{Species}$, the species diversity effect δ and the contributions of the pairwise species interactions $\delta_{Species\ 1*\ Species\ 2}$ to δ, were estimated using the diversity-interaction models developed by Kirwan *et al.* (2007; 2009). This was performed as in Ergon *et al.* (2016, Model 1), using weighted average concentrations and annual yield of nutritional components as Y. The estimated species and species interaction coefficients were used to estimate the effect of varying the species composition of the four species-seed mixture on NE_L yield, keeping the proportion of each species within the 0.1-0.7 range. The intra-annual stability of nutritional quality was assessed using a mixed models approach to estimate the variability of the responses across harvests within a year. This was also performed as described in Ergon *et al.* (2016, Model 2). All models were fitted using the GLM and MIXED procedures in SAS 9.2 (SAS Institute Inc., Cary, NC, USA.).

Results

Species diversity effects on nutritional composition of harvested forage

There were typical differences between legume and grass pure stands with legumes having higher concentrations of CP (163-2260 g (kg DM)$^{-1}$) and lower concentrations of NDF (342-438 g (kg DM)$^{-1}$) and WSC (136-187 g (kg DM)$^{-1}$) than grasses (90-130 g (kg DM)$^{-1}$, 453-544 g (kg DM)$^{-1}$ and 232-282 g (kg DM)$^{-1}$, respectively) (β coefficients in Table 1). The legume pure stands tended to have higher NE_L concentrations (5.89-6.31 MJ (kg DM)$^{-1}$) than grass pure stands (5.53-6.11 MJ (kg DM)$^{-1}$).

There was a significant diversity effect on ADF, WSC, NFC and NE_L concentrations in both cutting systems except in the 5-cut system in the second year (Table 1). There was also a significant diversity effect on NDF concentration in the 5-cut system in the first year. The effect was positive for NDF and ADF, leading to concentrations in the centroid mixture which was 7-10% higher than in the average pure stand (Table 2). For WSC, NFC and NE_L the diversity effect was negative, leading to a WSC concentration in a centroid mixture which was 20-22% lower, a NFC concentration which was 7-9% lower, and a NE_L concentration which was 4% lower than in the average pure stand. There was no significant species diversity effect on the CP concentration (Table 1). The diversity effects were not always transgressive (that is, having a higher value than the highest pure stand value) and it was therefore checked whether the observed diversity effects could be due to changes in species composition relative to the sown proportions (a sampling effect) by comparing the measured ADF, NDF, WSC, NCF and NE_L concentration with the concentrations that would be expected based on observed, rather than sown, species proportions in the mixtures (Table 3). The measured concentrations of ADF, NDF, WSC and NFC concentrations in both cutting systems, and of NE_L concentration in the 3-cut system, were still different from what would have been expected based on observed species proportions. This result indicated that the diversity effects on these variables were due to one or more species having different quality in mixtures than in pure stands. Analyses of nutritional quality in species-separated samples from the first and last harvests in the second year in the 3-cut system showed that the grass species had higher concentrations of CP, ADF and NDF, and lower concentrations of WSC, NFC and NE_L, when grown in mixtures compared with pure stands (Table 4). In the last harvest the CP concentration in the grasses was 40-50% higher in mixtures than in pure stands. ADF and NDF concentrations were 15-24 and 10-18% higher, and NE_L concentrations 8-10% lower, when the grasses were grown in mixtures as compared to pure stands. In the last harvest the two grass species differed in that the fibre and NE_L concentrations were not significantly affected in perennial ryegrass while they were in tall fescue. The concentrations of easily digestible carbohydrates (WSC, NFC) were much lower in mixtures than in pure stands for both grass species. At the first harvest the concentration of WSC was 32-41% lower and at the last harvest it was 69-76% lower in mixtures than in pure stands. In red clover, the CP concentration was not affected by stand type at all. For the carbohydrate fractions there was an opposite tendency to that seen in grasses, and in the last harvest the WSC concentration was significantly higher in mixtures than in pure stands. The results did not appear to be influenced by reproductive development, as tall fescue did not produce flowering stems in the last harvest, while red clover produced a lot of flowering stems, and perennial ryegrass a limited amount (data not shown). Sorted samples of white clover were not analysed due to limited white clover biomass in the samples from these harvests.

Species diversity effects on annual yields of crude protein and energy

Due to the strong positive and transgressive diversity effect on DM yield (Table 2 and Ergon *et al*. 2016), there were also significant positive and transgressive diversity effects on annual yields of nutritional components in both cutting systems and both years. The predicted annual NE_L yield of a centroid mixture was 52-72% higher than in the average pure stand (Table 2 and Supplementary File Table S3). The annual yield of CP is of particular interest, as it provides information on the N status of the swards. The predicted annual CP yield of a centroid mixture was 60-83% higher than in the average pure stand and 7-27% higher than in the red clover pure stand, which was the species with the highest CP yield. When we estimated the effect of varying the proportion of one species in the seed mixture from 0.1 to 0.7 while keeping the seed weight ratio between the three other species constant at 1:1:1 (Figure 1A), the maximum accumulated NE_L yield over the two years was estimated when the red clover proportion was 0.1 (both cutting systems). Together with the white clover this corresponds to a total legume proportion of 0.4. When we manipulated the white clover proportion, the maximum accumulated yield was obtained at a proportion of 0.1 in the 3-cut system and 0.4 in the 5-cut system, corresponding to a total legume proportion of 0.4 and 0.6. For perennial ryegrass the optimal proportions were 0.5 and 0.3 in the 3 and 5-cut systems, respectively, while for tall fescue it was 0.3 and 0.4. This corresponds to total legume proportions of 0.33 - 0.43. When we estimated the effect of varying the species seed weight ratios in all combinations, but keeping the minimum proportion of each species at 0.1, we found the maximum accumulated NE_L yield in the 3-cut system was obtained at proportions of 0.1 (red clover), 0.1

(white clover), 0.4 (tall fescue) and 0.4 (perennial ryegrass), while the maximum accumulated NE_L yield in the 5-cut system was obtained at proportions of 0.1 (red clover), 0.3 (white clover), 0.3 (tall fescue) and 0.3 (perennial ryegrass) (Figure 3B).

Intra-annual stability of nutritional quality

Mixtures had lower intra-annual variability than pure stands for NDF, ADF, WSC, NFC and all three energy concentrations in both the two first years, and for CP in the second year only ($\chi^2_{,6df}$ = 29.4-62.3, v *P* < 0.0001) (Figure 2).

Discussion

Differences between the species in CP, NDF, ADF and WSC content agreed with expectations. We found that there were significant species diversity effects on the nutritional composition of herbage from mixtures in the two first years of the 3-cut system and in the first year of the 5-cut system. Mixtures had lower WSC and NFC concentrations, higher ADF concentrations and slightly lower NE_L concentration than expected from the nutritional composition of pure stands. The diversity effects on WSC and ADF could not be explained by a change in species composition after sowing, indicating that changes in the nutritional composition in one or more species were involved. This was supported by the analysis of nutritional composition of single species sorted from the mixtures which were sampled from the first and last harvests in the 3-cut system the second year. These analyses showed that the grass species had higher concentrations of fibre and CP and lower concentrations of easily digestible carbohydrates and NE_L in mixtures than in pure stands. This may be an effect of higher availability of N, increased standing biomass, higher competition for light and lower leaf to stem ratios, in the mixtures as compared to the average pure stands (see Ergon *et al.,* 2016). Similarly, Gierus *et al.* (2012) found that perennial ryegrass had a higher NDF concentration and a lower NE_L concentration when grown together with lucerne than when grown in pure stand, and this coincided with a higher DM yield of the mixture. A reduction in concentrations of WSC, accompanied by an increase in concentrations of CP, was observed in perennial ryegrasses in mixtures with white clover by Evans *et al.* (1996). The difference between mixtures and pure stands was particularly pronounced during late summer, when clover growth was strongest. Evans *et al.* proposed that when growth demand for fixed carbon exceeds supply, such as when plants are shaded, WSC levels are reduced. Consequently, high summer yield of legumes in mixtures may result in a reduction in WSC content of the companion grass due to shading. No fertilizer was applied in the experiment reported by Evans *et al*., and differences in N availability and growth may therefore also have played a role. The lack of significant diversity effects on nutritional quality in the second year of the 5-cut system in our experiment may be related to the lower DM yield and less shading there. In addition, unlike in 2012, the harvests in the 5-cut system in 2011 were very unequal, with the second and fourth average mixture harvest comprising 32 and 37% of the annual harvest, respectively (data not shown). This may have caused a stronger diversity effect on nutritional quality in the 5-cut system in 2011 than if the harvests had been more evenly spaced.

Mixtures of grassland species have a higher light interception relative to pure stands (Spehn *et al.,* 2005) and grass species have longer leaves and shoots and invest more in supporting tissues and specific leaf area when grown in mixtures, particularly if legumes are present, indicating a role of N nutrition (Gubsch *et al.,* 2011). It is also known that N fertilization changes the chemical composition of plants; CP concentration increases and WSC concentration decreases while the effects on structural carbohydrates vary (Peyraud and Astigarrage, 1998; Hoekstra *et al.,* 2007). To what extent N fertilization has an effect on structural carbohydrates may depend on whether the plants are in a vegetative or reproductive growth stage. Calvière and Duru (1999) found an increase in the stem proportion of spring growth grass herbage with increasing N and P status. We found, however, that even in the absence of reproductive stems in the third harvest, tall fescue had significantly more structural carbohydrates when grown in mixtures than in pure stands with low N supply. We also observed that tall fescue grew longer leaves than perennial ryegrass in mixtures in the 3-cut system, which may have been associated with a higher proportion of ADF-rich supporting tissues in tall fescue. The negative effect of species diversity on nutritional quality in the grass component was not seen in red clover; in fact, species diversity tended to have the opposite effect. Unlike the other species, red clover may have experienced more competition for light in the pure stands than in mixtures, as the seeding rate used is high for legumes, and individual plants in pure stands can become very large. Higher levels of competition in pure stands may have caused a higher stem to leaf ratio and lower WSC concentration than in red clover plants grown in mixtures.

Sturludottir *et al.* (2014) studied mixtures of timothy, smooth meadow grass, white and red clover in Nordic and Canadian conditions at low N fertilization levels (40-80 kg ha^{-1}) and 2 cuts per year.

They analysed results across six sites and found strong diversity effects on DM yield over three years, but the diversity effect on nutritional quality was marginal. They did observe species interactions on nutrient variables, but these were both positive and negative and tended to cancel each other out. In contrast, we observed a net effect on several nutrient variables, which may be due to specific climatic or management conditions. Moreover, different grass species may vary in their responses to competition for light, their plasticity in stem to leaf ratios, and the differences in chemical composition between stems and leaves.

The diversity effect on nutritional quality observed in this study is likely to be associated with the low level of N-fertilization. Pure grass swards would normally receive higher levels of N fertilization than applied here, and this may be expected to have a similar effect on nutritional quality as cultivation in mixtures with legumes. However, although nutritional quality (in terms of the concentration of easily digestible carbohydrates) was somewhat lower in mixtures than in pure stands, there was still a very strong positive effect of diversity on NE_L and CP yield. This is mainly due to the strong positive diversity effect on DM yield (Ergon *et al.,* 2016). In addition, grasses absorb soil N efficiently, which has been shown to increase the symbiotic N acquisition of legumes (Nyfeler *et al.,* 2011). Estimations indicated that optimum seed weight proportions for accumulated DM yield over three years in four-species seed mixtures of red clover, white clover, perennial ryegrass and tall fescue was 0.1, 0.2, 0.4, 0.3 (3-cut system) and 0.1, 0.3, 0.3, 0.3 (5-cut system) (Ergon *et al*., 2016). Due to the effect of species diversity on NE_L concentration, the optimum proportions regarding accumulated NE_L yield over the two first years, studied here, shifted in favour of less white clover in the 3-cut system (0.1, 0.1, 0.4, 0.4). This likely reflects the stronger species diversity effect on nutritional quality in the 3-cut system than in the 5-cut system.

High intra-annual nutritional stability is often desired, especially in the context of practical farming. We found that the intra-annual nutritional stability was significantly higher in the average mixture than in the average pure stand in both cutting systems. This may partly be due to the fact that the four species have different seasonal patterns of stem formation and reproductive development. Previous research has shown that a combination of species in a mixture can result in a spread of herbage maturity through the year. For example, results from a mixture of three species (lucerne plus two grasses) grown in Argentina showed that although all three species were most mature in the summer, the grasses increased in maturity index during the spring, and lucerne extended its maturity through the autumn (Machado *et al*., 2007).

In conclusion, in our 3-cut system with low N fertilization, we found a significant species diversity effect on the chemical composition of forage harvested from mixed swards, leading to a 10% increase in ADF concentration, a 20-22% reduction in WSC concentration and a 4 % reduction in NE_L concentration averaged across the two first years. This diversity effect was at least partly due to reduced concentrations of WSC and increased concentrations of ADF in the grass component of mixtures compared with pure grass stands. An effect of species diversity in the 5-cut system was only found in the first year. We have also demonstrated that species diversity strongly reduces the intra-annual variability in nutritional quality at two different cutting frequencies.

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Supporting information

The following additional supporting information may be found in the online version of this paper

Table S1. Calibration and validation statistics for the ability of near-infrared spectroscopy to predict the nutritive attributes of the validation samples. CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; NFC, non-fibre carbohydrates, WSC, water-soluble carbohydrates, NE_L, net energy for lactation; DE, digestible energy; ME, metabolizable energy.

Table S2. A) Weighted annual concentrations (g (kg DM)$^{-1}$ or MJ (kg DM)$^{-1}$) and B) annual yields (t ha^{-1} or GJ ha^{-1}) of quality components measured in different sown stand types in the two first years after the sowing year. Lp, *Lolium perenne*; Fa, *Festuca arundinacea*; Tr, *Trifolium repens*; Tp, *T. pratense*; _p, pure stand; C, centroid (25 % seed weight of each species sown); _d, dominated (67 % of the indicated species, 11 % of each of the three other species sown). CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; WSC, water-soluble carbohydrates; NFC, non-fiber carbohydrates; NE_L, net energy for lactation. The averages of two replicate blocks are given.

Table S3. Parameter estimates for species identity coefficients β (LP, *Lolium perenne*; FA, *Festuca arundinacea;* TR, *Trifolium repens*; TP, *T. pratense*) and the species diversity coefficient δ, on the yield of net energy for lactation (NE_L, GJ ha^{-1}) and crude protein (CP, t ha^{-1}) in mixtures of the four species. The species identity coefficient equals the variable estimates in pure stands, while the species diversity coefficient equals the variable estimate in a centroid mixture minus the average of pure stands. All estimates were significant at $P<0.0002$.

Table 1. Parameter estimates for species identity coefficients β (LP, *Lolium perenne*; FA, *Festuca arundinacea;* TR, *Trifolium repens*; TP, *T. pratense*) and the species diversity coefficient δ, on nutritional variables in mixtures of the four species. The species identity coefficients equals the variable estimates in pure stands (g (kg DM)$^{-1}$ or MJ (kg DM)$^{-1}$), while the species diversity coefficient equals the variable estimate in a centroid mixture minus the average of pure stands. Estimates significant at P<0.05 are bolded. CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; WSC, water-soluble carbohydrates; NFC, non-fiber carbohydrates; NE_L, net energy for lactation.

			Nutritional variable					
Cutting system	Year	Parameter	CP	NDF	ADF	WSC	NFC	NE_L
3-cut	Year 1	β_{LP}	**103**	**488**	**280**	**282**	**326**	**5.94**
		β_{FA}	**95**	**544**	**320**	**232**	**275**	**5.53**
		β_{TR}	**214**	**375**	**290**	**144**	**317**	**6.06**
		β_{TP}	**199**	**380**	**307**	**136**	**322**	**5.91**
		δ	5	24	**28**	**-40**	**-25**	**-0.23**
	Year 2	β_{LP}	**109**	**507**	**301**	**246**	**305**	**5.80**
		β_{FA}	**90**	**508**	**289**	**277**	**309**	**5.81**
		β_{TR}	**163**	**438**	**293**	**187**	**307**	**5.93**
		β_{TP}	**178**	**395**	**305**	**170**	**325**	**5.89**
		δ	15	9	**28**	**-50**	**-21**	**-0.19**
5-cut	Year 1	β_{LP}	**109**	**507**	**302**	**241**	**298**	**5.79**
		β_{FA}	**115**	**515**	**301**	**238**	**290**	**5.75**
		β_{TR}	**226**	**369**	**276**	**141**	**320**	**6.23**
		β_{TP}	**204**	**363**	**291**	**149**	**338**	**6.12**
		δ	-4	**43**	**23**	**-44**	**-28**	**-0.23**
	Year 2	β_{LP}	**130**	**453**	**266**	**259**	**329**	**6.11**
		β_{FA}	**112**	**494**	**285**	**255**	**302**	**5.86**
		β_{TR}	**213**	**374**	**269**	**147**	**323**	**6.24**
		β_{TP}	**206**	**342**	**252**	**158**	**352**	**6.31**
		δ	9	4	-2	-13	-7	0.00

Table 2. Species diversity effects on nutritive components in a centroid mixture predicted from Model 1 and expressed as percent change relative to the value expected from the sown species proportions and the values of pure stands. Significance levels of the diversity effect estimates are given. Transgressive diversity effects are bolded. NDF, neutral detergent fibre; ADF, acid detergent fibre; WSC, water-soluble carbohydrates; NFC, non-fibre carbohydrates; NE_L, net energy for lactation; DM, dry matter; ***, P<0.0001; **, 0.0001<P<0.005; *, 0.005<P<0.05.

Nutritional variable	3-cut system		5-cut system	
	Year 1	Year 2	Year 1	Year 2
NDF (% of DM)	NS	NS	9 *	NS
ADF (% of DM)	10 ***	10 ***	7 **	NS
WSC (% of DM)	-20 **	-22 **	-21 **	NS
NFC (% of DM)	-8**	**-7***	**-9****	NS
NE_L (MJ kg^{-1} DM)	-4 **	**-4 ****	-4 **	NS
CP yield (t ha^{-1})	**76 *****	**83 *****	**60 *****	**68 ****
NE_L yield (GJ ha^{-1})	**52*****	**56*****	**68*****	**72*****
DM yield (t ha^{-1})	**56*****	**90*****	**64*****	**94*****

Table 3. Measured concentrations of nutritional components (g (kg DM)$^{-1}$ or MJ (kg DM)$^{-1}$) as compared to expected concentrations, based on concentrations in pure stands and either sown species proportions, or observed species proportions. Species proportions were visually observed for each plot before each harvest and an annual average weighted for DM yield was calculated. Averages across all mixture types and replicates (N=10). NDF, neutral detergent fibre; ADF, acid detergent fibre; WSC, water-soluble carbohydrates; NFC, non-fibre carbohydrates; NE_L, net energy for lactation. *, significantly different from the measured concentration (LSD, P<0.05).

			Nutritional variable					
Cutting system	**Year**		**CP**	**NDF**	**ADF**	**WSC**	**NFC**	**NE_L**
3-cut	Year 1	Measured	156	468	322	167	290	5.67
		Exp. sown	155	444	298*	196*	309*	5.86*
		Exp. observed	149	440	298*	207*	317*	5.88*
	Year 2	Measured	145	470	318	183	294	5.71
		Exp. sown	134	464	297*	220*	310*	5.85*
		Exp. observed	141	441*	304*	218*	318*	5.84*
5-cut	Year 1	Measured	160	471	310	159	290	5.80
		Exp. sown	164	433*	289*	193*	316*	6.01*
		Exp. observed	146	424*	277*	197*	304*	5.73
	Year 2	Measured	172	420	267	194	321	6.13
		Exp. sown	166	401	261*	210	332*	6.18
		Exp. observed	163	407	258*	218*	335*	6.20*

Table 4. Concentration of nutritive components (g (kg DM)$^{-1}$ or MJ (kg DM)$^{-1}$) in species grown in pure and in mixed stands at the first and third harvest in the second year of the 3 cut system. Significance levels are given when the quality of a species grown in mixture was different from the same species grown in pure stand. Lp, *L. perenne;* Fa, *F. arundinacea*; Tp, *Trifolium pratense;* CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; WSC, water-soluble carbohydrates; NFC, non-fiber carbohydrates; NE_L, net energy for lactation; DE, digestible energy; ME, metabolisable energy; ***, P<0.0001; **, 0.0001<P<0.005; *, 0.005<P<0.05.

				Nutritional variable					
Species[1]	**Harvest**	**Species composition**	**N[2]**	**CP**	**NDF**	**ADF**	**WSC**	**NFC**	**NE_L**
Lp	First	Pure stand	2	96	458	241	349	366	6.3
	First	Mixture	10	114	524*	298*	236*	293*	5.8*
	Third	Pure stand	2	113	585	372	139	256	5.5
	Third	Mixture	8	157*	583	388	33*	239*	5.4
Fa	First	Pure stand	2	82	520	284	314	318	5.9
	First	Mixture	10	111	573*	328*	186*	249*	5.4*
	Third	Pure stand	2	78	530	318	249	307	5.8
	Third	Mixture	9	120*	625**	390**	78**	217*	5.2*
Tp	First	Pure stand	2	234	302	237	141	374	6.4
	First	Mixture	7	232	289	221	162	386	6.5
	Third	Pure stand	1	159	457	407	86	284	5.3
	Third	Mixture	10	158	406	355	161**	317	5.6

[1]White clover was not analysed due to limited biomass in the samples
[2]Samples from both replicate blocks and five mixtures with different relative sown species proportions were included, unless when there was not enough biomass of the species in the sample.

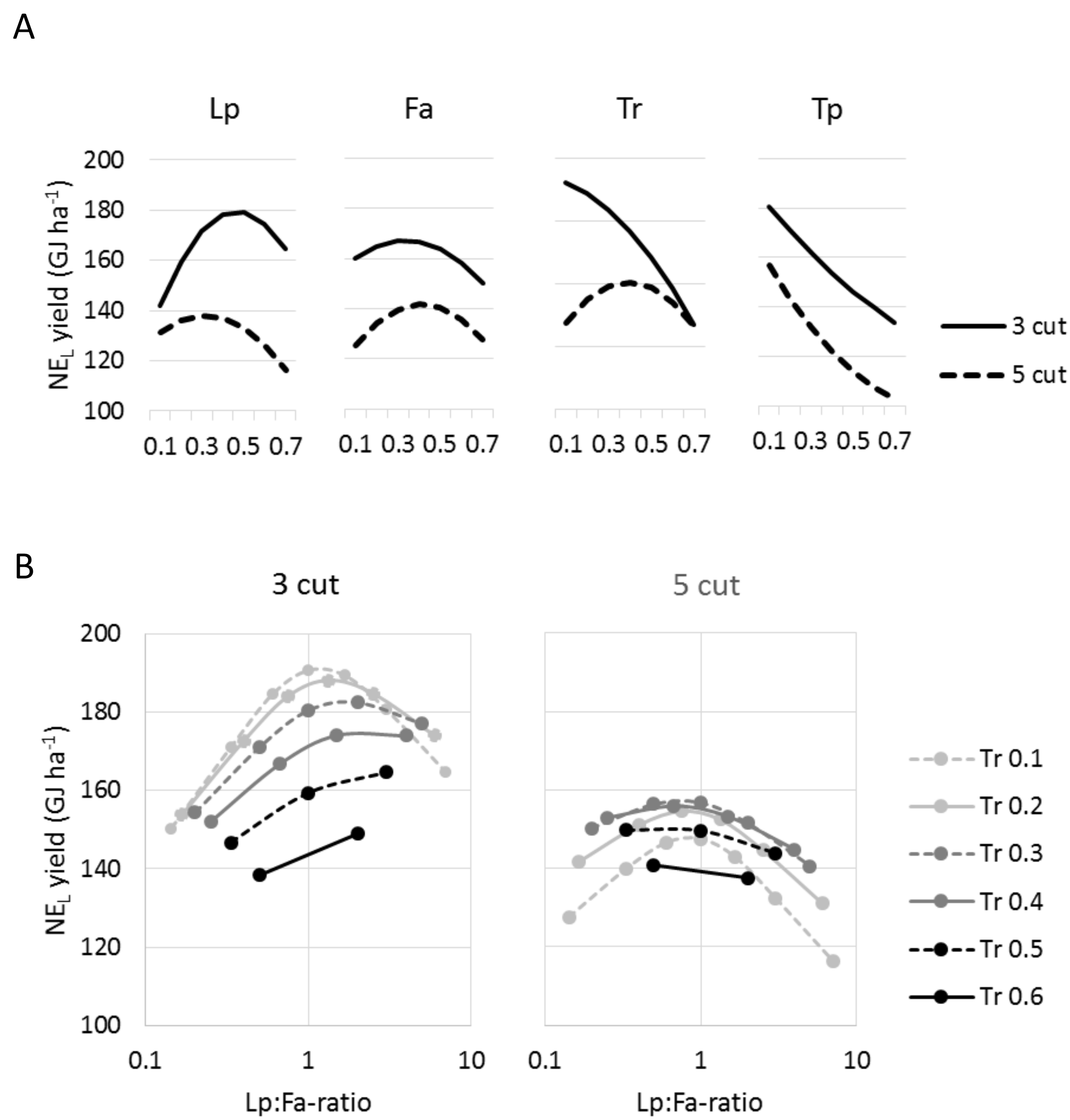

Figure 1. Estimation of total NE_L yield accumulated over the two first years in a 3 cut and a 5 cut system as a response to sown species composition (proportions of seed weight), using estimated species identity and species interaction coefficients for each species pair, cutting system and year. Lp, *Lolium perenne*; Fa, *Festuca arundinacea*; Tr, *Trifolium repens*; Tp, *T. pratense*. A) The proportion of the indicated species was varied from 0.1 to 0.7, keeping the ratios between the three other species constant at 1:1:1. B) The proportion of Tp was kept at 0.1 and the proportion of Tr was varied from 0.1 to 0.6. The ratio between Lp and Fa (x-axis, logarithmic scale) was varied within each level of Tr or Tp proportion.

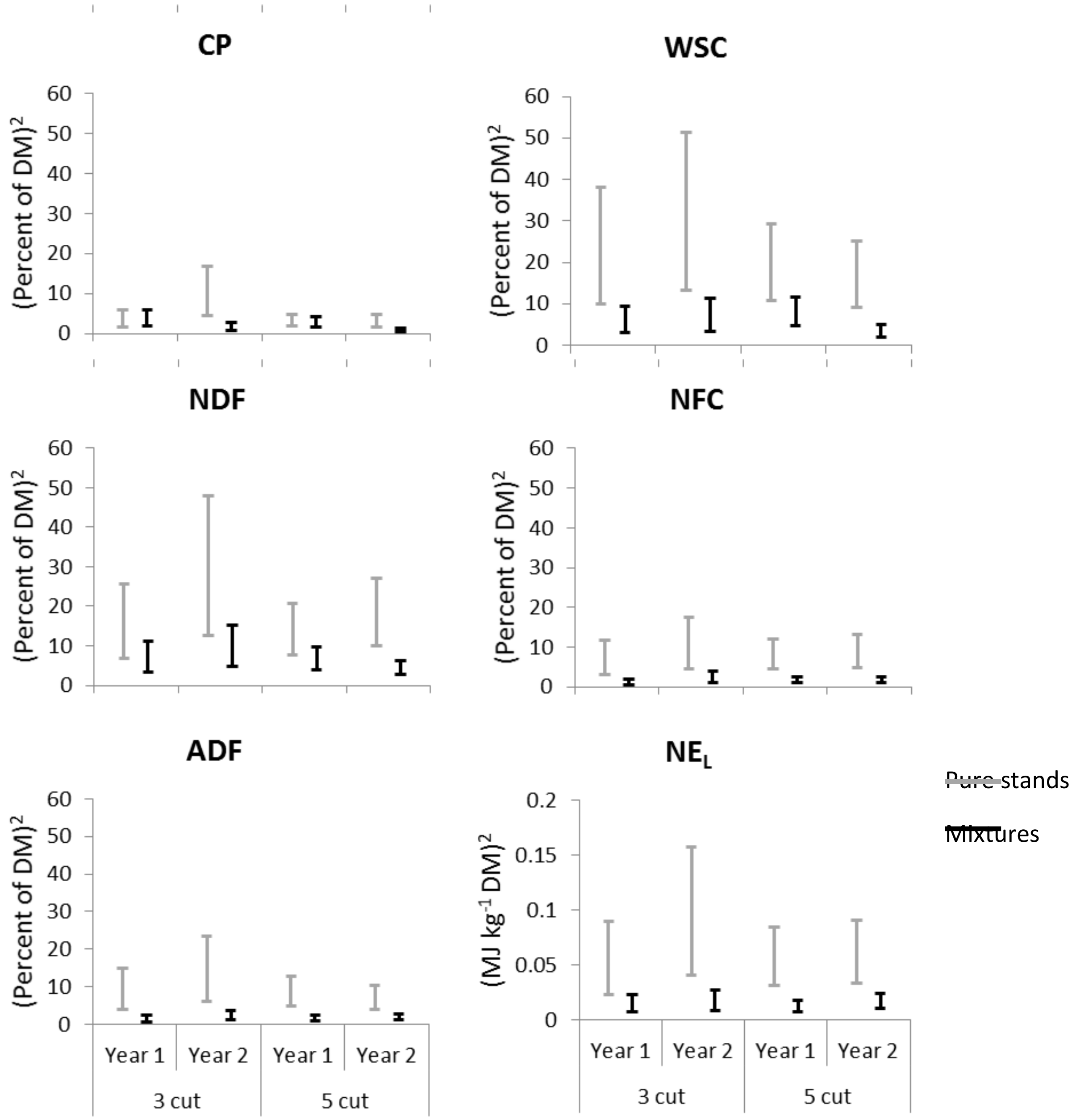

Figure 2. Confidence intervals of o the mean (95 %) for the intra-annual variability of nutritional components in the harvested pure stands of *Lolium perenne, Festuca pratensis, Trifolium repens* and *T. pratense* (grey) or mixtures of these species (black) in the two first harvesting years (Y1 and Y2) in a 3 cut system and a 5 cut system. The intra-annual variability was estimated as variance components according to Model 2. CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; WSC, water soluble carbohydrates; NFC, non-fiber carbohydrates, NE$_L$, net energy for lactation.

Supporting information

Table S1. Calibration and validation statistics for the ability of near-infrared spectroscopy to predict the nutritive attributes of the validation samples. CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; NFC, non-fibre carbohydrates, WSC, water-soluble carbohydrates, NE_L, net energy for lactation; DE, digestible energy; ME, metabolizable energy.

Variable	N	Mean	St. dev.	Est. min	Est. max	R^2
CP (g (kg DM)$^{-1}$)	56	166	59	0	344	0.99
NDF (g (kg DM)$^{-1}$)	60	413	94	130	697	0.97
ADF (g (kg DM)$^{-1}$)	60	167	44	142	406	0.97
NFC (g (kg DM)$^{-1}$)	59	325	42	200	449	0.87
WSC (g (kg DM)$^{-1}$)	60	196	75	0	421	0.98
NE_L (MJ kg^{-1} DM)	60	6.1	0.4	4.9	7.3	0.87
DE (MJ kg^{-1} DM)	58	12.1	0.5	10.5	13.7	0.90
ME (MJ kg^{-1} DM)	58	10.3	0.5	8.7	12.0	0.90

Table S2. A) Weighted annual concentrations (g (kg DM)$^{-1}$ or MJ (kg DM)$^{-1}$) and B) annual yields (t ha^{-1} or GJ ha^{-1}) of quality components measured in different sown stand types in the two first years after the sowing year. Lp, *Lolium perenne*; Fa, *Festuca arundinacea*; Tr, *Trifolium repens*; Tp, *T. pratense*; _p, pure stand; C, centroid (25 % seed weight of each species sown); _d, dominated (67 % of the indicated species, 11 % of each of the three other species sown). CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; WSC, water-soluble carbohydrates; NFC, non-fiber carbohydrates; NE_L, net energy for lactation. The averages of two replicate blocks are given.

A)

Year	Cutting	Species	CP	NDF	ADF	WSC	NFC	NE_L
2011	3-cut	Lp_p	94	495	277	296	327	59
		Fa_p	84	559	322	240	271	55
		Tr_p	232	349	284	131	323	61
		Tp_p	207	366	305	133	327	59
		C	170	434	307	169	304	58
		Lp_d	144	478	316	192	297	57
		Fa_d	141	497	330	174	279	56
		Tr_d	157	474	326	162	283	56
		Tp_d	167	457	329	140	287	56
	5-cut	Lp_p	95	526	303	254	295	57
		Fa_p	104	529	302	246	287	57
		Tr_p	246	339	271	125	325	63
		Tp_p	209	357	292	144	340	61
		C	161	477	314	151	287	58
		Lp_d	155	472	310	163	290	58
		Fa_d	153	486	311	172	285	58
		Tr_d	159	483	309	161	286	58
		Tp_d	175	435	305	145	303	59
2012	3-cut	Lp_p	96	530	304	255	301	58
		Fa_p	81	514	290	289	309	58
		Tr_p	176	423	290	172	307	60
		Tp_p	191	375	302	162	330	59
		C	157	460	323	165	296	57
		Lp_d	152	453	314	185	300	57
		Fa_d	134	484	310	197	294	57
		Tr_d	137	481	318	194	293	57
		Tp_d	146	469	325	174	294	57
	5-cut	Lp_p	111	473	265	281	328	61
		Fa_p	102	505	287	267	299	58
		Tr_p	229	354	268	129	325	63
		Tp_p	217	327	250	145	356	64
		C	172	413	262	196	326	62
		Lp_d	185	405	268	185	324	62
		Fa_d	160	444	273	202	311	61
		Tr_d	170	432	269	196	314	61
		Tp_d	173	406	262	193	329	61

B)

Year	Cutting	Species	CP	NDF	ADF	WSC	NFC	NE_L
2011	3-cut	Lp_p	0.99	5.24	2.93	3.10	3.44	62.52
		Fa_p	0.86	5.77	3.32	2.47	2.79	56.55
		Tr_p	1.43	2.18	1.77	0.81	2.00	38.08
		Tp_p	1.92	3.40	2.83	1.24	3.04	55.16
		C	2.35	6.01	4.26	2.34	4.21	80.39
		Lp_d	2.00	6.65	4.39	2.67	4.12	79.43
		Fa_d	1.92	6.74	4.48	2.35	3.78	75.35
		Tr_d	1.82	5.55	3.81	1.90	3.31	65.89
		Tp_d	2.01	5.49	3.95	1.68	3.44	67.70
	5-cut	Lp_p	0.77	4.26	2.46	2.05	2.38	46.48
		Fa_p	0.76	3.87	2.21	1.80	2.10	41.73
		Tr_p	1.52	2.08	1.66	0.77	2.00	38.85
		Tp_p	1.97	3.36	2.75	1.35	3.20	57.64
		C	1.91	5.67	3.72	1.80	3.40	68.47
		Lp_d	1.81	5.48	3.60	1.89	3.37	67.27
		Fa_d	1.75	5.59	3.56	1.98	3.27	66.19
		Tr_d	1.78	5.41	3.46	1.79	3.21	65.07
		Tp_d	1.96	4.91	3.44	1.64	3.41	66.03
2012	3-cut	Lp_p	0.54	2.95	1.67	1.50	1.73	32.79
		Fa_p	0.74	4.70	2.65	2.64	2.83	52.95
		Tr_p	1.34	3.35	2.28	1.37	2.38	46.26
		Tp_p	2.27	4.47	3.62	1.91	3.94	70.70
		C	2.27	6.66	4.66	2.40	4.28	82.35
		Lp_d	2.06	6.13	4.25	2.51	4.06	77.75
		Fa_d	1.79	6.67	4.23	2.69	3.96	77.79
		Tr_d	1.65	5.77	3.82	2.32	3.52	68.46
		Tp_d	2.06	6.59	4.57	2.46	4.15	79.88
	5-cut	Lp_p	0.37	1.57	0.88	0.93	1.08	20.07
		Fa_p	0.65	3.22	1.83	1.71	1.91	37.14
		Tr_p	1.57	2.44	1.84	0.89	2.23	43.19
		Tp_p	1.63	2.45	1.87	1.08	2.67	47.60
		C	1.66	3.98	2.53	1.88	3.14	59.46
		Lp_d	1.56	3.42	2.26	1.56	2.74	52.06
		Fa_d	1.64	4.55	2.80	2.07	3.18	62.02
		Tr_d	1.75	4.44	2.77	2.02	3.24	62.88
		Tp_d	1.56	3.66	2.36	1.74	2.96	55.34

Table S3. Parameter estimates for species identity coefficients β (LP, *Lolium perenne*; FA, *Festuca arundinacea;* TR, *Trifolium repens*; TP, *T. pratense*) and the species diversity coefficient δ, on the yield of net energy for lactation (NE_L, GJ ha^{-1}) and crude protein (CP, t ha^{-1}) in mixtures of the four species. The species identity coefficients equals the variable estimates in pure stands, while the species diversity coefficient equals the variable estimate in a centroid mixture minus the average of pure stands. All estimates were significant at *P*<0.0002.

Cutting system	Year	Parameter	CP yield	NE_L yield
3-cut	Year 1	β_{LP}	**1.09**	**63.42**
		β_{FA}	**0.95**	**57.14**
		β_{TR}	**1.35**	**39.05**
		β_{TP}	**1.79**	**52.82**
		δ	**0.98**	**27.53**
			0.77	**47.38**
	Year 2	β_{LP}	**0.81**	**43.30**
		β_{FA}	**1.20**	**40.63**
		β_{TR}	**2.09**	**55.34**
		β_{TP}	**1.01**	**26.10**
		δ	**0.89**	**38.19**
			0.86	**53.56**
5-cut	Year 1	β_{LP}	**1.45**	**44.49**
		β_{FA}	**1.87**	**67.96**
		β_{TR}	**0.76**	**34.57**
		β_{TP}	**0.52**	**22.14**
		δ	**0.77**	**39.38**
			1.52	**44.35**
	Year 2	β_{LP}	**1.48**	**44.50**
		β_{FA}	**0.73**	**27.08**
		β_{TR}	**1.09**	**63.42**
		β_{TP}	**0.95**	**57.14**
		δ	**1.35**	**39.05**