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Quality Legume-Based Forage Systems for Contrasting Environments - COST 852

Einsatz von Leguminosen als Basis hoher Grundfutterqualität unter verschiedenen Umweltbedingungen

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Preamble

COST 852 “Quality Legume-Based Forage Systems for Contrasting Environments” has been very successful and has attracted a large group of scientists from more than 20 countries all over Europe. Within this COST action comprehensive field experiments have been established in several European countries to investigate and improve the role of forage legumes in grassland. COST 852 supplies benefits for society by providing high quality agricultural products coupled with reduced environmental impact. It also provides information to policy makers on the pertinence of using legume-based systems with an added benefit of increased self-sufficiency from agricultural production within Europe.

The benefits to agriculture centres on the development of more reliable systems. The action improves the selection of appropriate legume species/cultivars and the development of regionally adapted pasture management practices in contrasting environments of Europe. This ultimately leads to more secure and sustainable systems for forage and livestock production.

The benefits to the scientific community include stimulation of active communication between scientists involved at all levels of research on the development and utilisation of forage legumes in Europe. This will promote a multidisciplinary approach to the study of complex agricultural systems. It will also provide researchers with access to a range of climatic environments, which may occur in any individual country but not reliably enough for experimental purposes.

COST 852 – final meeting at AREC Raumberg-Gumpenstein 2006

1. Introduction

In most European countries there is currently considerable emphasis on low input, efficient agricultural systems that reduce production costs, promote environmental production policy and maintain a living and open countryside. The sustainability of these production systems strongly relies on the cultivation of forage legumes due to their ability to contribute to the nitrogen economy of swards through nitrogen fixation, their high feeding value and their ability to improve and maintain soil structure.

Within Europe half the annual requirement for feed is provided by grassland. However, although the EU is a net exporter of feed grain it is a substantial importer of protein and non-grain feed ingredients. The amount of raw material imported for feed corresponds to the production from 10 million ha of land. The current production of meat, eggs and milk relies on the importation of non-forage protein and this represents 27% of the total amount of protein consumed by the animal. Although non-forage proteins of vegetable origin are available, much of the 'by-pass proteins' have traditionally come from animal by-products and fish meal. Imported feed-components have high transportation costs, high environmental impact and their quality and safety can be highly variable. A greater reliance on 'home-grown' legume-based protein sources would improve the traceability of the feed, enhance consumer confidence in the final market product and promote ecologically sound farming systems.

As farming within the north-western European countries moves towards less intensive forms of agriculture, it has been predicted that there will be a growth in intensive agricultural systems in the Baltic States and other Central and Eastern European countries in order to supply consumer demand in central EU countries. However, the growth of industrial forms of livestock production has been associated with serious environmental and safety problems.

Consequently, forage legumes, adapted to a wide range of soil types, climatic conditions and management systems, will become increasingly important components of sustainable agriculture production systems in Europe. Legume-based systems are known to contribute towards sustainable, environmentally sensitive and energy efficient agriculture and are likely to assume an increasing importance. However, the use of legumes in grassland systems has not yet increased markedly. World-wide, the estimate for annual N_2 fixation on agricultural soils is about 90Mt of N, of which 56% is fixed by legumes. However, N_2 fixation in European countries is estimated to be much less. The interest in ecological forms of agriculture has increased substantially in recent years and presently organic farming accounts for 2.9% of agricultural land within the EU. Many EU countries are actively encouraging the uptake of organic farming systems and this will inevitably increase farmers' reliance on legumes. Recent results also suggest that elevated levels of CO_2 in the atmosphere and increasing temperatures associated with global warming will improve the ability of legumes to fix atmospheric nitrogen. This is likely to substantially increase the effectiveness of legume-based systems.

Temporal and spatial variation in legume performance occurs and this restricts the confidence of farmers in legume-based systems. If reliability is to be improved and the range of forage legumes extended in Europe we will require understanding of the constraints of environment, the reasons for divergence between species' potential and actual performance, the causes of yield variability and lack of persistence, the mechanisms controlling diet selection in animals and the role of management. Understanding the mechanisms underlying nutrient flows in ruminants fed on legume-based diets is an essential prerequisite for the achievement of high animal performance coupled with high efficiency and reduced environmental impact. Such information is essential for an improvement in nitrogen use efficiency.

2. Material and methods

COST 852 was subdivided into three main activities (i) legume genetic resources, (ii) sward management and (iii) forage utilization. AREC Raumberg-Gumpenstein officially joined COST 852 at the end of 2001 by signing the expression of interest. The reporter was nominated the national responsible and was also invited to participate in the management committee of COST 852.

2.1 Field experiment

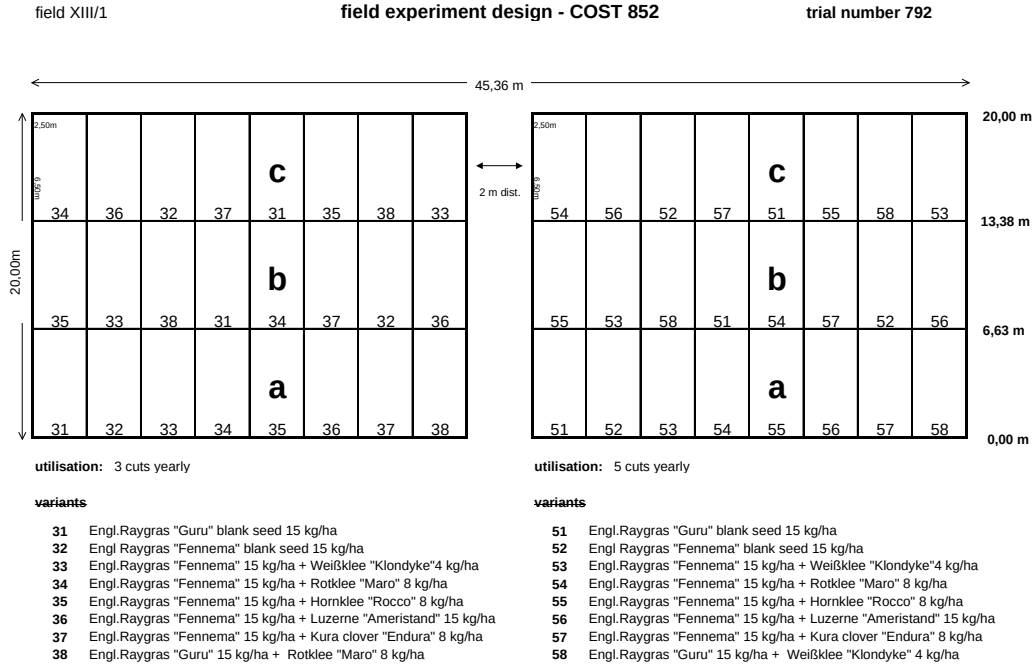

Figure 1: Field experiment design of COST 852 at AREC Raumberg-Gumpenstein

The activities of AREC Raumberg-Gumpenstein focused on working group 3 which covered aspects of (i) animal intake and grazing behaviour, (ii) quality of legume-based fresh and ensiled forage and (iii) the mechanisms of N-flows within the ruminant (efficiency-losses). Two field trials including different legumes resp. legume/grass mixtures combined with a varying utilisation frequency have been established and analysed on the basis of a common core protocol. This field experiment (figure1) has been carried out for 3 complete vegetation periods from 2003 until 2005, which is to be seen the minimum duration time for such objectives. Both field trials consisted of each 8 variants randomized in three replications with a single plot size of 16.25 m². Block 1 was utilized by three cuts per year ("silage cutting"), whereas Block 2 was harvested five times per year, simulating a grazing system.

Beside the *lolium perenne* species "Fennema", which was used by all international partners, the Austrian field design was extended and also included the new breeding "GURU", that is a successful output of the internal breeding activities of AREC Raumberg-Gumpenstein. The introduced field experiments have been carried out in a close cooperation with some partners of working group 3 (esp. University of Kiel, Institute of Crop Science and Plant Breeding - Grass and Forage Science/Organic farming; ETH Zürich - Institute of Plant Science and Institute of Animal Science; Agricultural University of Norway, Dept. of Horticulture and Crop Sciences).

2.2 Analyses and recordings

The following parameters were analysed, recorded respectively estimated within the field respectively laboratory experiments:

2.2.1 Environmental parameters

Air temperature (2 m above soil surface, °C), global radiation (J m^{-2}) and precipitation (mm) were regularly measured and recorded at the weather station of AREC Raumberg-Gumpenstein, which is located in a distance of about 300m of the experimental plots. All relevant weather data are measured automatically in short intervals and are online transmitted to the ZAMG (Central institute of meteorology and geodynamics at Vienna), where the date are checked for plausibility and aggregated for feasible datasets.

2.2.2 DM yield

The field plots were cut three respectively five times per year with a mowing machine. The yield of fresh mass was weighed and a representative sample was taken to analyse the dry matter content and to assess the dry matter yield.

2.2.3 Botanical composition

The phenological stage of plants at the time of cutting was recorded using the following code: grass vegetative (gv), with elongated stems (gs), with visible ears (ge), flowering (gf) legumes (w,r,l,b) vegetative (e.g. for red clover: rv), with elongated stems (rs) with visible buds (rb), flowering (rf). A sample of 550 g FM was taken from the yield of every single plot and was manually separated into the functional groups of grasses, legumes and herbs.
Beside this separation (only for the 3-cut system) the number of legume plants was counted in all field plots of all variants. This was done during the first growth (three times of recording) in all three years.

2.2.4 Contents of N, CP, ADF, NDF, ADIN (acid detergent insoluble nitrogen), lignin

N was analysed by a rapid combustion (850°C), conversion of all N products into N_2, subsequent measurement by thermo-conductivity cell (elementar analysator Vario Max CN, Germany) and expressed as CP (N*6,25). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were analysed according to VAN SOEST et al. (1991) using a semiautomatic fibre analyser (ANKOM fibre analyser, USA). NDF was assayed without a heat stable amylase and NDF and ADF expressed including residual ash.

2.2.5 Energy content by Pepsin-cellulase

The pepsin-cellulase method (CM) was carried out according to De Boever et al. (1986) in Germany, following the guidelines of VDLUFA Standard Methodology (VDLUFA, 1993). The procedure involves a preliminary incubation for 24 h with pepsin/HCl at 40°C, followed by 45 min heating at 80°C and a second incubation with a commercial cellulase Onozuka R-10 from Trichoderma viride (Merck, Darmstadt, Germany). The metabolisable energy content (ME) was computed applying the estimating equation for legumes of Weissbach et al. (1996) on the values obtained with the in vitro procedure:

(1) $ME\ (MJ/kg\ DM) = 13.98 - 0.0147*XA - 0.0137*IOM + 0.00234*CP$

where XA is the crude ash content (g/kg DM), IOM is enzymatically insoluble organic matter (g/kg DM), and CP is crude protein content (g/kg DM)

Results of the standard samples were calculated as cellulase digestibility of the organic matter (CDOM; % DM), based on the calculated loss upon ashing (L; g), initial weight of the sample (W; g), dry matter content (DM; %) and ash content (XA; %) (VDLUFA, 1993):

(2) $CDOM\ (\%\ DM) = 100 - (L * 1\ 000\ 000) / (W * DM * (100 - XA))$

where L is calculated loss upon ashing (g), W is initial weight of the sample (g), DM is dry matter content (%) and XA is ash content (%)

2.2.6 Energy content by In-vitro digestibility

The two-stage method by Tilley and Terry (1963) (TT) was carried out with some Austrian modifications. Rumen fluid was obtained from two rumen-fistulated oxen, fed with a diet of seasonal green forage from mixed swards and supplemented with concentrates. The buffer solution was dispensed according to McDougall (1948). Before incubation, rumen fluid and buffer solution were mixed in the proportion 1:4 (v/v). Pepsin solution was prepared by dissolving 20 g of 1:10,000 pepsin (Sigma-Aldrich, Germany) in 1000 ml distilled water. Where required, steps were carried out under anaerobic conditions, flushing buffers and solutions with gaseous CO_2. Dried forage samples (0.5 g) were weighed in triplicate into 100 ml Erlenmeyer flasks and 50 ml of the rumen liquor-buffer solution were added. Remaining air was expelled with CO_2 and flasks sealed with perforated Parafilm, followed by incubation of samples and blanks at 38.5°C for 48 h in the dark incubator. After the end of the first incubation period, pH value was adjusted to 1.5 units by using 2.2N HCl, 5 ml of pepsin solution were added and then the flasks were incubated for 48 h at 38.5°C again. After the end of the second incubation period, samples were filtered (Macherey Nagel MN 640w, Germany), dried at 104°C for 4 hours and weighed before ashing at 450°C.

The digestibility of organic matter (OMD, %) was calculated as difference between the OM of the sample before incubation and the residual OM. Residues after incubation measured in blanks were deducted. Extreme outliers were excluded from further calculations. Estimated in vitro digestibility values of the standard samples were compared within each run to their corresponding in vivo values by linear regression. A correction equation for in vitro values of the samples in terms of variability due to rumen fluid quality between runs and expressing results of the samples as estimated in vivo OMD was generated. ME content for the samples in the present experiment was estimated by regression analysis of analysed DOM (g/kg DM) with standard values obtained from the DLG guidelines (DLG, 1997). Regressions were performed separately for the first cut and subsequent regrowths, giving the following equations:

(3) 1^{st} cut: ME (MJ/kg DM) = 0.0174 * OMD - 1.2677

(4) regrowths: ME (MJ/kg DM) = 0.0172 * OMD - 1.0306

For the comparison of the performance of the two in vitro methods values of the standard samples were used in the present experiment without routine correction and expressed as DOMTT (%).

2.2.7 NIRS analysis

To gain a complete dataset for all forage samples, ME, ADF, NDF and N were estimated by near-infrared reflectance spectroscopy (NIRS), thereby calculating the energy contents based on every of the two in vitro methods. All samples available were scanned twice using a NIR-Systems 5000 scanning monochromator (Perstrop Analytical Inc., Silver Spring, MD, USA), where software (ISI-version) for data collection and manipulation was supplied by Infrasoft International® (ISI, Port Matilda, PA, USA). Absorbance was recorded as log (1/reflectance) = log (1/R) at 2 nm intervals throughout the near-infrared region (1100-2500 nm) to give a total of 700 data points. Prior to the calibration process samples were checked for erroneous measurements and outliers, using the option ‘centre samples’ of the ISI® software, which provides a ranking of the spectral data on the basis of the standardized Mahalanobis distance (H) from the average spectrum. Samples with H-values exceeding 3.0 were excluded from the calibration procedure. The option ‘select samples’ on the basis of H-value 0.6 was used to select calibration subsets which represented the whole sample spectrum while the validation subsets were randomly selected after ranking the spectral data according to their H distance. Calibrations were developed by regressing laboratory determined values against the NIR

spectral data, using the Modified Partial Least-Squares (MPLS) method (Shenk and Westerhaus, 1991) with or without scatter correction for particle size. The minimum F statistics for terms included in the equation was 8.0.

2.3 Statistical analysis

Statistical analyses were performed using the software package SAS 9.1 (SAS Institute Inc., 2004). A mixed model analysis was calculated for energy content data (ME) of each defoliation system using PROC MIXED by considering site (available for the silage cutting system), year, cut, species, and method as fixed factors. Cuts were treated as repeated measurement and assuming a symmetric covariance structure. Effects were considered significant in all statistical calculations for P-values <0.05. In case of significant interactions, linear contrasts were calculated using the SLICE procedure of SAS. Comparison of least squares means were performed by t-test with a Bonferroni-Holm adjustment of previously defined contrasts. The relationship between the NIRS-estimated ME contents based on the two different in vitro methods was determined by linear regression analysis using the REG procedure of SAS. Parameters of the determined regression equations (slope, intercept) were tested whether they differ from unity or zero respectively.
For additional statistical and descriptive analysis of yield productivity and yield quality SPSS was also applied using ANOVA and comparison of means.

2.4 Abbreviations

ADF, acid detergent fiber; CDOM, cellulase digestibility of organic matter; CM, pepsin-cellulase method; CP, crude protein; DM, dry matter; DOM, digestible organic matter; DOMTT, digestible organic matter determined by Tilley and Terry procedure; ME, metabolisable energy; MECM, metabolisable energy determined by pepsin-cellulase method; METT, metabolisable energy determined by Tilley and Terry procedure; NDF, neutral detergent fiber; NIRS, near-infrared reflectance spectroscopy; OM, organic matter; OMD, organic matter digestibility; RMSE, root mean square error

3. Results and discussion

Some of the results were exclusively worked out for this final report whereas some other have been published in the meantime and are cited as original chapters. Gratitude has to be expressed to Dr. Birgit Eickler, Dr. Martin Gierus and Prof. Dr. Friedhelm Taube from the CAU at Kiel who very actively were involved in COST 852 and strongly interacted with AREC Raumberg-Gumpenstein.

3.1 Yield productivity and forage quality of different legumes – results of the Austrian COST 852-experiments

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3.1.1 DM yield

According the common protocol of working group 3 the dates of harvest were defined by the growth time between the cuts which was aimed at 50 days for the 3-cut experiment and 30 days for the 5-cut experiment. The real growth time was 55 days (s=1.2) respectively 33 days (s=7.2). Due to the different yearly conditions (length of the winter period, begin of the vegetation time) the first growth was harvested between 22nd of May and 1st of June, which meets the situation in agricultural practice very well.

There were significant differences between the treatments concerning dry matter production within the two cutting systems (figure 2). In the pure grass stands the average yield amounted to 47 resp. 49 dt dry matter per ha and year which represents the natural potential of the site. The effect of legumes as an important companion plant for grasses is impressively demonstrated by much higher yields for the combination of ryegrass with white clover, red clover and bird's foot trefoil. The most productive and stable combination during the whole period was ryegrass with white clover both in the 3-cut and in the 5-cut experiment. Bird's foot trefoil performed sufficiently in the 3 cut system, whereas lucerne showed a disappointing performance at all, mainly caused by a low pH-value (5.0) and by insufficient soil phosphorus content (4 mg P per 100g fine soil). With the exception of ryegrass in combination with red clover and bird's foot trefoil, which are sensitive to higher utilisation frequency, there were no significant differences in the yield production between the two cutting regimes. These results indicate the limitation of yield production by the site conditions, which in practice lead to a typical 3 cut system with possibly one additional grazing activity in favourable years.

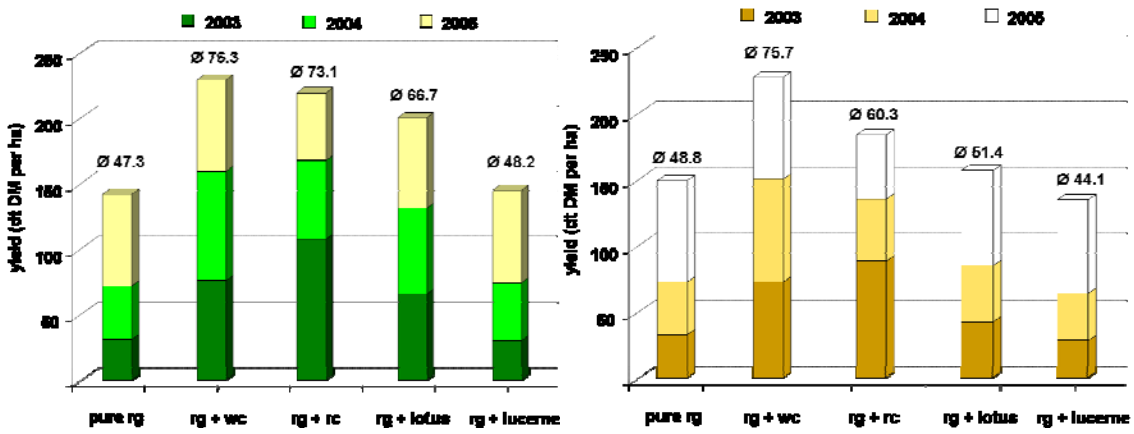

Figure 2: Yield productivity during the observation period (left chart = 3 cut system, right chart = 5 cut system)

The different colours within the columns are representing the yield of the different years which were significantly influenced by the type of the mixture. The yield of the pure grass stands increased over the years, whereas the mixture of white clover and rye grass showed a steady yield situation during the experimental period. Red clover in combination with rye grass performed best in the first year, whereas the mixtures with bird's foot trefoil and lucerne showed a yield increase in the third year. In general there was only little yield difference between the growths in the 3-cut system (Ø 20.9 dt DM ha^{-1} 1st cut, Ø 22.0 dt DM ha^{-1} 2nd cut, Ø 18.1 dt DM ha^{-1} 3rd cut). In the 5-cut system the first growth yielded best with Ø 21.2 dt DM ha^{-1} followed by the 2nd growth with Ø 13.3 dt DM ha^{-1} whereas the 3rd, 4th and 5th growth showed low yields (< 9 dt DM ha^{-1}). From an economical point of view these low yielding growths have to be critically considered for their cost-benefit ratio.

In addition to the common protocol the Austrian ryegrass cultivar "Guru" was implemented in all cutting regimes as pure grass and in combination with red clover (3-cut system) respectively with white clover (5-cut system). This Austrian cultivar performed worse than the cultivar "Fennema" which according to the common protocol was used by all partners but it has to be considered that the breeding goals of "Guru" were mainly focusing on endurance and winter hardness. An investigation period of three years is certainly too short to highlight these important long term properties.

By means of the manual separation of representative forage samples into grasses, legumes and herbs the total yield of each variant could be related to these functional groups of which legumes are of highest interest (Figure 3). In the first year the yield of the pure ryegrass stand was not influenced by any legumes, whereas in 2004 and 2005 white clover invaded the plots and increasingly contributed to the yield productivity. White clover kept a very stable proportion of around 55 to 60% all over the observed period and underlined its important role for grassland. Red clover highly contributed to the yield in the first year but dropped down continuously to less than 30%. Bird's foot trefoil, which is seen to be a low competitive legume species, has been quite persistent for the first two years and even, kept a proportion of one third in 2005.

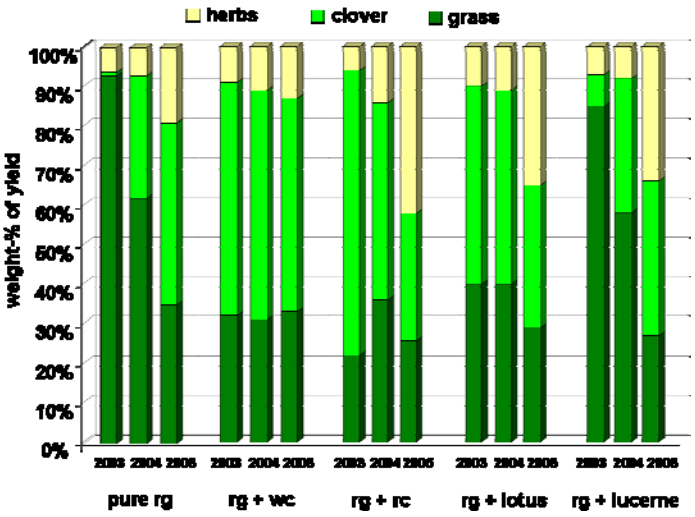

Figure 3: Relative yield proportion of grass, legumes and herbs during the observation period from 2003 - 2005

In the first year lucerne established poorly but showed an increasing proportion during the following two years. In general the results demonstrate a good contribution of different legumes to the productivity of grassland, which did not receive any additional nitrogen fertilizer. Biological N-fixation of legumes which has been investigated by several studies all

over the world plays an important role in low input farming systems both on grassland and arable land (HELGADOTTIR and PÖTSCH, 2007). The use of this natural nitrogen source allows reducing external nitrogen input which causes high costs and environmental problems (TAUBE and PÖTSCH, 2001; PÖTSCH, 2007). The proportion of (unsown) herbs increased in all treatments during the observation period, whereas grasses and legumes developed variably. Generally the proportion of legumes in the observed treatments has been reflected in the yield productivity of the different mixtures (PÖTSCH and RESCH, 2007).

3.1.2 Forage quality (analysed according to 2.2.6)

Table 1, 1a, 2 and 2a include the results of the different legumes and of ryegrass (both from the blank seed and from the mixtures) of the three-cut system for the years 2003, 2004 and 2005 (Forage from the five-cut system was not analysed for quality parameters). In all years the digestibility of organic matter of the legume samples was low with the exception of red clover and lucerne which were within the range of the data given in the German feeding value table (DLG-Futterwerttabelle 1997) that is also used in Austria for feeding calculations. This is mainly caused by the relatively long growth period, which leads to higher contents of hardly digestible or indigestible substances in plants (EICKLER et al., 2007a, 2007b).

Bird´s foot trefoil showed significantly lower digestibility values than all other legumes, which might have been caused by a higher concentration of condensed tannins (GIERUS et al., 2007). There was just little impact of the companion legumes on the quality of ryegrass in the mixtures. The energy concentrations for the analysed samples are relatively low compared with DLG data and show great differences within the tested legumes (Table 2).

Table 1: Digestibility of Organic Matter (DOM %) – average of three cuts/year

	white clover	red clover	bird´s foot trefoil	lucerne	ryegrass (blank seed)	ryegrass (from mixtures)
2003	71.4[a]	68.6[a]	57.8[b]	65.0[a]	70.5[a]	71.5[a]
s	6.0	4.9	4.6	5.8	4.1	4.6
2004	72.4[a]	66.0[a]	57.4[b]	62.8[b]	72.4[a]	74.0[a]
s	3.3	4.8	4.3	6.3	3.1	5.3
2005	74.9[a]	64.0[b]	55.3[c]	57.4[c]	78.4[a]	76.3[a]
s	6.7	6.4	2.9	3.9	2.8	4.3
DLG	80 -81	61-79	n.a.	57-75	68-83	n.a.

Table 1a: Analysis of variance - depending variable - Digestible Organic Matter (DOM%)

source	Square sum type III	df	average square	F	significance
corrected modell	11028,100(a)	17	648,712	28,820	,000
constant term	853550,070	1	853550,070	37920,778	,000
year	1,774	2	,887	,039	,961
variant	9721,412	5	1944,282	86,379	,000
year * variant	1108,983	10	110,898	4,927	,000
error	5672,210	252	22,509		
total	1345835,879	270			
corrected total variation	16700,310	269			

a R-square = .660 (corrected R-square = .637)

Forage quality of white clover was significantly higher than that of all other legumes. Red clover, used in many Austrian seed mixtures for permanent grassland and ley-farming areas showed medium to low energy concentration, but was significantly better than bird´s foot trefoil and lucerne which had inferior values. There was a positive impact of legumes on the quality of the companioned resulting in higher energy concentration in 2003 and 2004.

Table 2: Energy concentration (MJ Net Energy Lactation/kg DM) – average of three cuts/year

	white clover	red clover	bird´s foot trefoil	lucerne	ryegrass (blank seed)	ryegrass (from mixtures)
2003	5.8[ab]	5.5[ab]	4.3[c]	5.1[b]	5.8[ab]	5.9[a]
s	0.67	0.53	0.52	0.65	0.46	0.53
2004	6.0[ab]	5.2[bc]	4.3[d]	4.9[cd]	6.0[a]	6.3[a]
s	0.36	0.53	0.49	0.71	0.35	0.57
2005	6.2[a]	5.0[b]	4.0[c]	4.3[bc]	6.8[a]	6.5[a]
s	0.76	0.72	0.33	0.45	0.51	0.53
DLG	6.5-7.1	5.0-6.9	n.a.	5.1-6.3	5.5-7.1	n.a.

Table 2a: Analysis of variance - depending variable – energy concentration (MJ NEL/kg DM)

source	Square sum type III	df	average square	F	significance
corrected modell	155,873(a)	17	9,169	31,358	,000
constant term	5529,457	1	5529,457	18910,909	,000
year	,086	2	,043	,148	,863
variant	137,138	5	27,428	93,803	,000
year * variant	15,364	10	1,536	5,255	,000
error	73,684	252	,292		
total	9172,372	270			
corrected total variation	229,556	269			

a R-square = .679 (corrected R-square = .657)

3.1.3 Quality yield

The product of dry matter yield and energy concentration results in the energy yield (MJ NEL/ha), which is presented in figure 4. The pure, unfertilised grass stand amounted to 15.7 GJ NEL/ha and year, which is comparable with the data of extensively managed alpine and mountainous grassland in Austria. The highest energy yield per ha and year resulted from the mixture of ryegrass and red clover in 2003 with more than 55 GJ NEL, which is within the range of ley farming areas and intensively used grassland. Mixtures with ryegrass and white clover or red clover showed the best overall productivity for the three years period followed by mixtures with ryegrass and bird´sfoot trefoil or lucerne.

For agricultural practice it is therefore of great importance to use white or red clover in seed mixtures with companion grass(es) to achieve both high yield amounts and sufficient forage quality. Under the given site conditions bird´sfoot trefoil and lucerne moderately contributed to the total yield but showed disappointing low values of digestibility and energy concentration which resulted in low quality yields at all.

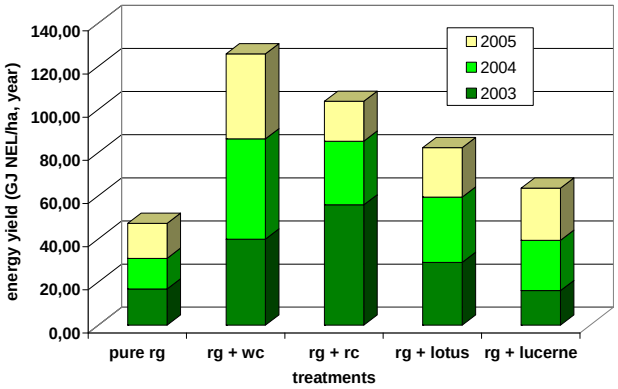

Figure 3: Quality yield (GJ NEL/ha) during the total observation period from 2003 – 2005

3.1.4 Botanical composition

Beside the separation of the yields of the 3-cut system into grass, legumes and herbs (shown under 3.1.1, figure 3) the number of legume plants was counted in all field plots of all variants. This was done at three different times during the first grow up in all three years of the field experiments.

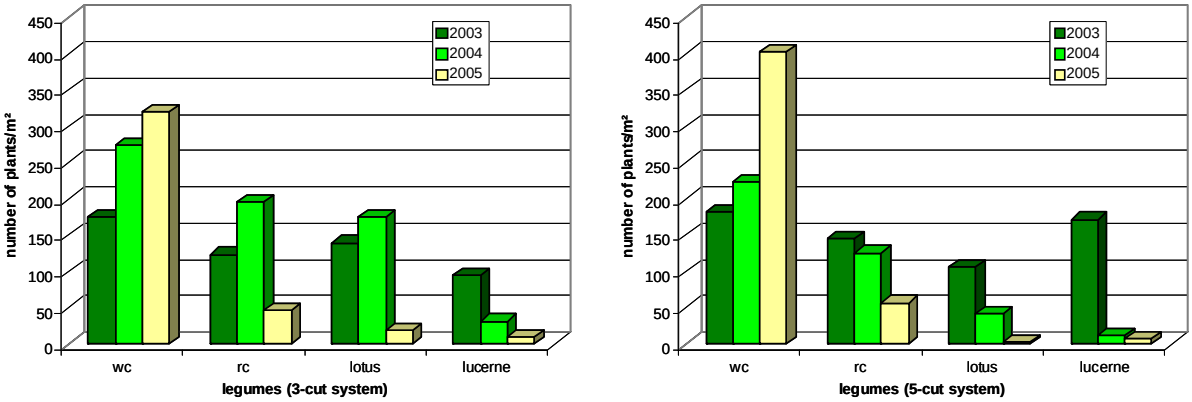

Figure 4: number of legume plants/m² during the total observation period from 2003 - 2005

Figure 4 shows the development of legume plants in the different binary mixtures with ryegrass. White clover was the most competitive legume and increased steadily over the years in both cutting systems. It is well known that white clover tolerates quite high amounts of nitrogen and is also able to perform under a high frequency of utilization (cutting and grazing) due to its ability of building stolons.

Red clover established and grew sufficiently in the first two years but decreased dramatically in the year 2005. This might be caused by the missing adaptation of the used cultivar to the harsh conditions of the mountainous site at AREC Raumberg-Gumpenstein (average yearly temperature is 6.8°C in combination with a short vegetation period and long periods with snow cover). This could also be the reason for the very similar development of bird´sfoot trefoil which established quite well in the 3-cut system for the first two years, whereas the higher cutting frequency led to a strong decrease after the first year. Lucerne established sufficiently in 2003 but could not stand over the total experiment period.

Summary and conclusion of 3.1

According to the common protocol of working group 3 within COST 852 two field experiments have been carried out at AREC Raumberg-Gumpenstein, Austria. Comprehensive recordings and analyses have been made during 3 years focussing on yield

productivity, forage quality and botanical composition of the established mixtures. Special attention was given to different legume species and on their function for grassland ecosystems. The results of these experiments clearly demonstrate the function and importance of legumes for grassland ecosystems. In most cases the grass/legume mixtures performed significantly better than the pure grass stands. But there were considerable differences between the investigated binary grass/legume mixtures concerning dry matter yield, competitiveness, forage quality and energy yield production. All these aspects have to be considered for the selection of legumes and for their usage in seed mixtures. Concerning the relatively low digestibility and energy concentration values, optimal grass and legume cultivars have to be chosen, which are well adapted to the given site conditions.

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3.2 Estimation of the energy content of several forage legumes based on two different *in vitro* methods

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3.2.1 Introduction

Considerable emphasis currently exists on the research for agronomy and utilisation of forage legumes. Agronomic research is focused on farm and regional scale as well as on a wide range of climatic and management regimes, as increasingly important components of sustainable agriculture production systems in Europe are taking place, driven by the Nitrates Directive (Council Directive 91/676/EEC, 1991) and the Drinking Water Directive (Council Directive 98/83/EC, 1998). Farmers are forced to reduce nitrogen overload, and forage legumes therefore become an interesting alternative for the substitution of mineral nitrogen fertilizer. Interdisciplinary approaches regarding forage legume resources can be realised particularly in specific multi-site programmes (Wachendorf et al., 2004), involving the evaluation of quality legume-based forage systems for contrasting environments.

Due to the complex structure of such network programmes, the demand for reliable and efficient analyses for the examination of extensive sample sets is obligatory for comprehensive interpretations, e.g. in case of forage quality parameters. Recognised standard methods for estimation of OMD are the Tilley and Terry procedure (TT), and the pepsin-cellulase method (CM). Their advantages and limitations for application were evaluated and thoroughly discussed for a range of forages in numerous articles and reviews (Ayres, 1991; Weiss, 1994; Stern et al., 1997; Kitessa et al., 1999; Gosselink et al., 2004). In general, the main feature of the CM method is the independency of donor animals and its repeatability within and between runs. The variability in the rumen fluid quality is one of the disadvantages of the TT procedure, whereas possible interaction between microbial species in the rumen and tested forages occurring during TT cannot be reproduced by the CM method. However, reported results of studies are inconsistent on that score, as contrary findings related to highest accuracy are shown for rumen-fluid techniques compared to cellulase-based methods (Aufrère and Michalet-Doreau, 1988; De Boever et al., 1988; Givens et al., 1989; Givens et al., 1990; Steg et al., 1990), or a similar potency to predict OMD was proved (Gosselink et al., 2004).

Overall, for various types of forages prediction of OMD is higher with CM than with chemical methods and comparable to results obtained by in vitro methods with rumen microorganisms (Aufrère and Guérin, 1996). Calculation of ME content is generally based on estimation equations, developed for the respective methods and different feedstuffs to perform best results. For the CM, a regression equation was developed separately for legumes based on in vivo digestibility trials by Weissbach et al. (1996).

The NIR method is limited by the quality of the reference analytical methods and the quality of appropriate estimation equations. Thus, the question remains if the currently existing estimation methods and equations which are available as specified prediction tools in animal nutrition are sufficient for a wide range of different legume species, grown as binary legume-grass mixtures, under different environmental conditions and various management systems. Therefore, unlike in defined research approaches to investigate systematically effects of

forages on the quality of analysis methods, the methods should be robust enough to cover a range of different samples and their variation, which may result from differences in sampling, sample preparation (e.g. cutting, drying, grinding), or other factors during the preparation process.

To investigate whether the two mentioned in vitro methods, the Tilley and Terry procedure (TT) and the pepsin-cellulase method (CM), are adequate and sufficient as a basis for a reliable estimation of ME contents of different forage legumes, we compared the results gained from the analysis of a set of samples. We hypothesized that due to its mentioned features the CM is more robust and thus the ME estimation based on this method may be more reliable.

3.2.2 Material and methods (see 2.25, 2.2.6 and 2.2.7)

3.2.3 Results

Table 3 shows the descriptive statistics of the NIRS calibration results for all samples included in the evaluation, giving the range of estimated values for ME contents of the respective methods (MECM; METT).

Table 3: NIRS calibration statistics of the determined parameters IOM (g/kg DM), ME_{CM} (MJ/kg DM), TTDOM (%), ME_{TT} (MJ/kg DM), N (%), NDF (%) and ADF (%) of the legume samples (n = 431)

Parameter	n (1)	range (2)		mean (3)	SD (4)	SEC (5)	R^2 (6)	CVSD (7)
IOM	77	90.60	353.73	204.42	72.18	11.52	0.98	15.96
ME_{CM}	76	8.18	11.90	10.11	0.93	0.15	0.97	16.27
TTDOM	77	42.42	79.83	63.51	7.82	3.24	0.83	41.43
ME_{TT}	73	6.20	11.03	8.61	1.05	0.59	0.69	55.86
N	75	1.62	5.24	3.70	0.74	0.09	0.99	11.76
NDF	77	25.79	61.43	44.62	9.33	2.49	0.93	26.73
ADF	77	17.69	44.07	26.64	7.95	1.91	0.94	24.01

(1) Number of samples included in the calibration
(2) Minimum and maximum of the parameter values
(3) Mean of the parameter values
(4) Standard deviation of the laboratory-determined values
(5) Standard error of calibration
(6) Coefficient of determination; relationship between NIRS- and laboratory-determined values
(7) Variation coefficient referred to the SD of the reference method ($CV_{SD} = SEC*100/SD$)

Additionally, the variation coefficient referred to the standard deviation (CVSD=SEC*100/SD) was calculated in order to assess the suitability of the respective method for reliable NIRS calibration, as suggested by Murray (1986). If a product shows a narrow range in composition, or if the error in estimation is large compared with the spread (as SD) in composition, then regression gets increasing difficulty in finding stable NIR calibrations. Where the error exceeds one-third of the SD of the whole sample spectrum, regression can be misleading.

The NIRS calibration models obtained correlations with all nutrient and energy variables analysed with R2>0.90, with exception of METT with a considerably lower R2 of 0.69, compared to MECM (R2=0.97). The less precise prediction of METT was reflected in a SEC four times larger than for MECM, resulting in a CVSD of 56% and thus exceeding the

acceptable level, whereas all other parameters mentioned gained CVSD values within this limit. The calibration model for METT is thus classified as poor. Estimation of the in vitro parameters resulted in a more precise prediction of IOM (R^2=0.98), whereas the TTDOM had a lower R^2 (0.83), with a CVSD value of 41%.

Table 4 shows four standard samples as well as the descriptive statistics of their in vitro analyses, calculated as differences of the in vitro analysed DOM to their in vivo digestibility (dCDOM, Cellulase method; dDOMTT, Tilley and Terry procedure, uncorrected data). Based on CM, the CDOM of three standard samples were strictly overestimated (mean dCDOM 2.1%…3.8%), whereas one standard sample was underestimated (mean dCDOM -3.6%). Differences of uncorrected DOMTT for the standard samples included a wide range of differences within each standard sample, with a poorer accuracy indicated by remarkably large negative differences up to 15.6%.

Table 4: Differences of the *in vitro* analysed DOM (dCDOM: Cellulase method; $dDOM_{TT}$: Tilley and Terry) of four standard samples to their *in vivo* digestibility; $dDOM_{TT}$ data prior to routine correction with *in vivo* DOM

Standard samples			*In vivo* DOM	dCDOM (%)					$dDOM_{TT}$ (%)				
No.	Cuts/year	Cut	(%)	n (1)	range (2)		mean (3)	S.D. (4)	n (1)	range (2)		mean (3)	S.D. (4)
198	1	1	41.9	5	0.72	3.30	2.06	1.13	24	-15.44	1.89	-2.44	3.99
232	3	1	68.4	5	-3.91	-2.88	-3.63	0.43	24	-10.82	1.09	-3.45	3.16
246	4	2	75.2	4	2.92	4.34	3.78	0.63	24	-7.74	0.93	-3.50	3.07
298	1	1	46.7	5	2.65	3.65	2.98	0.40	23	-15.56	1.22	-3.20	4.68

(1) Number runs in which standard samples were included
(2) Minimum and maximum of the differences to the *in vivo* value
(3) Mean of the differences to the *in vivo* value
(4) Standard deviation of the differences of the laboratory-determined values to the *in vivo* values

Means of dDOMTT ranged from -2.4 to -3.5%, indicating a general underestimation by TT, with a comparably high SD. For this reason, the routine analysis by TT procedure was amended by a standard correction procedure prior to further statistical evaluation, as described before. Thus, a less precise estimation remains, as shown by regression analysis of ADF and ME contents (Figure 5).

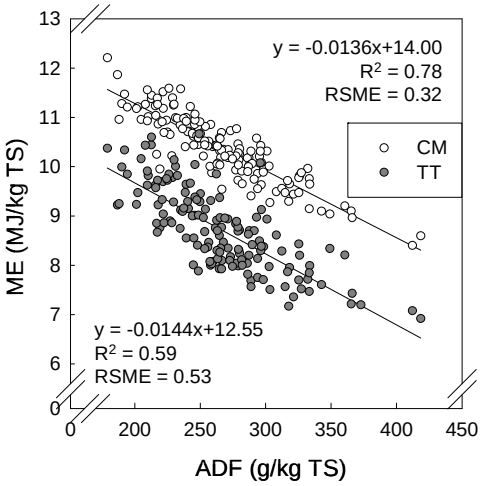

Figure 5: Relationship between ME and ADF content of several forage legumes estimated by NIRS based on Cellulase method (CM) respectively Tilley and Terry (TT). Graphs include all data of both study sites and years (if available) as means over field replicates (n=149)

Results of the statistical analysis are shown in Table 5 for the silage cutting system including both study sites and in Table 6 for the study site Germany subdivided into defoliation systems. The factor 'method' consistently represented the largest variance component ($P<0.05$) in all datasets. As the 3-way interaction between the main factors cut, species, and method were not significant, the respective 2-way interactions of these factors are presented in the following.

Table 5: Influence of the factors 'year', 'site', 'cut', 'species' and 'method' on the ME contents of different forage legumes. Statistical analysis of data from Germany and Austria, silage cutting system, years 2004 and 2005 (n = 286)

	ME	
Effect	DF	F value
replicate(year)	4	3.08 *
site	1	1.8 ns
year	1	5.92 *
site*year	1	0.64 ns
cut	2	154.91 ***
site*cut	2	95.8 ***
year*cut	2	23.59 ***
site*year*cut	2	15.81 ***
species	3	283.4 ***
site*species	3	7.94 ***
year*species	3	1.01 ns
site*year*species	3	18.49 ***
cut*species	6	6.6 ***
site*cut*species	6	4.18 ***
year*cut*species	6	1.93 ns
site*year*cut*species	6	4.03 **
method	1	1590.99 ***
site*method	1	32.45 ***
year*method	1	13.81 ***
site*year*method	1	0.36 ns
cut*method	2	6.11 **
site*cut*method	2	7.05 **
year*cut*method	2	8.02 ***
site*year*cut*method	2	0.46 ns
species*method	3	14.57 ***
site*species*method	3	1.26 ns
year*species*method	3	2.3 ns
site*year*species*method	3	2.17 ns
cut*species*method	6	0.64 ns
site*cut*species*method	6	1.29 ns
year*cut*species*method	6	1.49 ns
site*year*cut*species*method	6	0.55 ns

Levels of significance: * for $P<0.05$; ** for $P<0.01$; *** for $P<0.001$; ns for $P\geq0.05$

Table 6: Influence of the factors 'year', 'cut', 'species' and 'method' on the ME contents of several forage legumes subjected to different defoliation systems. Statistical analysis of data from Germany, years 2004 and 2005

	silage cutting (n = 176)		simulated grazing (n = 276)		rotational grazing (n = 238)	
Effect	DF	F value	DF	F value	DF	F value
replicate(year)	4	3.65 *	4	0.98 ^{ns}	4	1.92 ^{ns}
year	1	0.04 ^{ns}	1	205.90 ***	1	1.95 ^{ns}
cut	2	119.28 ***	4	210.22 ***	4	45.59 ***
year*cut	2	28.41 ***	4	53.57 ***	4	43.97 ***
species	4	94.93 ***	4	459.80 ***	3	578.62 ***
year*species	4	7.26 ***	4	11.29 ***	3	15.66 ***
cut*species	8	5.22 ***	16	14.52 ***	12	12.69 ***
year*cut*species	8	2.44 *	16	7.19 ***	12	2.12 *
method	1	490.67 ***	1	7774.50 ***	1	5027.68 ***
year*method	1	2.1 ^{ns}	1	17.21 ***	1	43.61 ***
cut*method	2	9.38 ***	4	10.30 ***	4	18.01 ***
year*cut*method	2	5.21 **	4	18.13 ***	4	6.36 ***
species*method	4	6.18 ***	4	52.04 ***	3	42.76 ***
year*species*method	4	2.05 ^{ns}	4	0.46 ^{ns}	3	0.50 ^{ns}
cut*species*method	8	0.88 ^{ns}	16	1.12 ^{ns}	12	1.06 ^{ns}
year*cut*species*method	8	0.73 ^{ns}	16	0.53 ^{ns}	12	1.87 ^{ns}

Levels of significance marked with * for $P<0.05$; ** for $P<0.01$; *** for $P<0.001$; ^{ns} for $P\geq0.05$

Results of the statistical evaluation of the 2-way interactions are given in Table 7 for the data from the silage cutting system at both study sites, and in Table 8 for the data of the study site Germany, separated for each defoliation system. In general, consequently lower ME values were estimated based on TT compared to CM, with LSMeans in each dataset for the respective defoliation systems significantly different (P<0.05) between methods within species, and between methods within cut. For the dataset including both study sites (Table 7), significantly higher ME values as means over cuts were consistently estimated based on the respective method for white clover, followed by red clover.

Table 7: ME contents of several forage legumes from the silage cutting system at three cutting dates as means over study sites and years, estimated by NIRS based on two different *in vitro* methods (n = 286)

	Cellulase method				Tilley and Terry				Means over methods		
	Cut 1	Cut 2	Cut 3	*Mean*	Cut 1	Cut 2	Cut 3	*Mean*	Cut 1	Cut 2	Cut 3
White clover	11.0	10.1	10.6	10.6 ^{v,V}	10.1	8.8	9.4	9.4 ^{w,V}	10.5 ^{a,A}	9.4 ^{a,C}	10.0 ^{a,B}
Red clover	10.3	9.7	10.0	10.0 ^{v,W}	9.2	8.2	8.8	8.7 ^{w,W}	9.8 ^{b,A}	8.9 ^{b,C}	9.4 ^{b,B}
Lucerne	9.9	9.5	9.5	9.6 ^{v,X}	8.3	7.6	7.8	7.9 ^{w,X}	9.1 ^{c,A}	8.5 ^{c,B}	8.6 ^{c,B}
Birdsfoot trefoil	9.9	9.3	9.4	9.5 ^{v,X}	8.5	7.6	7.5	7.9 ^{w,X}	9.2 ^{c,A}	8.5 ^{c,B}	8.4 ^{d,B}
Mean	10.3 ^{g,G}	9.6 ^{g,I}	9.9 ^{g,H}	9.9	9.0 ^{h,G}	8.1 ^{h,I}	8.4 ^{h,H}	8.5			

a,b,c,d LSMeans are significantly different between species within cutting date at $P<0.05$; $SE = 0.07$
A,B,C LSMeans are significantly different between cutting dates within species at $P<0.05$; $SE = 0.07$
g,h LSMeans are significantly different between methods within cutting date at $P<0.05$; $SE = 0.05$
G,H,I LSMeans are significantly different between cutting date within method at $P<0.05$; $SE = 0.05$
v,w LSMeans are significantly different between methods within species at $P<0.05$; $SE = 0.05$
V,W,X,Y LSMeans are significantly different between species within method at $P<0.05$; $SE = 0.05$

Lowest ME values were estimated for lucerne and birdsfoot trefoil. As means over species, forage of the 1st cut showed highest ME values, and lowest in the 2nd cut. However, the range

of ME for the species was only slightly larger within the METT dataset (1.5 MJ) than within MECM (1.1 MJ). Averaged over methods, ME values of these species did not differ between the 2nd and 3rd cut, but ME of birdsfoot trefoil was significantly lower in the 3rd cut (8.4 MJ).

Table 8: ME contents of forage legumes from different defoliation systems (silage cutting, SC; simulated grazing, SG; rotational grazing, RG) from the site Germany as means over years, estimated by NIRS based on two different *in vitro* methods

	Cellulase method						Tilley and Terry						Means over methods				
	Cut 1	Cut 2	Cut 3	Cut 4	Cut 5	*Mean*	Cut 1	Cut 2	Cut 3	Cut 4	Cut 5	*Mean*	Cut 1	Cut 2	Cut 3	Cut 4	Cut 5
Silage cutting (n = 176)																	
WC	11.0	10.0	10.2			10.4 ^{v,V}	10.6	8.8	9.3			9.6 ^{w,V}	10.8 ^{a,A}	9.4 ^{a,C}	9.8 ^{a,B}		
RC	10.4	9.7	9.4			9.8 ^{v,W}	9.6	8.3	8.6			8.8 ^{w,X}	10.0 ^{c,A}	9.0 ^{b,B}	9.0 ^{b,B}		
KC	10.8	10.3	10.3			10.5 ^{v,V}	9.9	9.0	8.9			9.2 ^{w,W}	10.3 ^{b,A}	9.6 ^{a,B}	9.6 ^{a,B}		
LC	9.8	9.5	9.0			9.4 ^{v,X}	8.4	7.7	7.4			7.8 ^{w,Y}	9.1 ^{e,A}	8.6 ^{c,B}	8.2 ^{c,C}		
BT	10.0	9.7	8.9			9.6 ^{v,X}	9.1	7.9	7.0			8.0 ^{w,Y}	9.6 ^{d,A}	8.8 ^{bc,B}	8.0 ^{c,C}		
Mean	10.4 ^{g,G}	9.8 ^{g,H}	9.6 ^{g,I}			9.9	9.5 ^{h,G}	8.3 ^{h,H}	8.2 ^{h,H}			8.7					
Simulated grazing (n = 276)																	
WC	11.8	11.1	9.8	11.1	11.2	11.0 ^{v,V}	10.1	9.9	8.6	9.7	9.5	9.5 ^{w,V}	10.9 ^{a,A}	10.5 ^{a,B}	9.2 ^{b,C}	10.4 ^{a,B}	10.4 ^{a,B}
RC	11.2	10.8	9.9	10.4	10.7	10.6 ^{v,X}	9.6	9.5	8.4	8.8	8.6	9.0 ^{w,X}	10.4 ^{b,A}	10.1 ^{b,B}	9.1 ^{b,D}	9.6 ^{c,C}	9.6 ^{c,C}
KC	11.4	10.9	10.5	10.9	10.8	10.9 ^{v,W}	9.6	9.5	9.0	9.4	9.2	9.3 ^{w,W}	10.5 ^{b,A}	10.2 ^{b,B}	9.7 ^{a,C}	10.1 ^{b,B}	10.0 ^{b,B}
LC	10.8	10.4	10.0	10.1	10.4	10.4 ^{v,Y}	8.8	8.6	8.0	8.2	8.2	8.4 ^{w,Y}	9.8 ^{c,A}	9.5 ^{c,B}	9.0 ^{b,D}	9.2 ^{d,CD}	9.3 ^{d,BC}
BT	10.9	9.9	9.8	10.1	10.4	10.2 ^{v,Z}	8.5	7.9	7.6	7.9	8.0	8.0 ^{w,Z}	9.7 ^{c,A}	8.9 ^{d,C}	8.7 ^{c,D}	9.0 ^{c,C}	9.2 ^{d,B}
Mean	11.2 ^{g,G}	10.6 ^{g,HI}	10.0 ^{g,J}	10.5 ^{g,I}	10.7 ^{g,H}	10.6	9.3 ^{h,G}	9.1 ^{h,H}	8.3 ^{h,J}	8.8 ^{h,I}	8.7 ^{h,I}	8.8					
Rotational grazing (n = 238)																	
WC	11.5	10.9	10.6	11.2	11.5	11.1 ^{v,V}	10.5	9.7	9.5	9.7	9.4	9.8 ^{w,V}	11.0 ^{a,A}	10.3 ^{a,B}	10.0 ^{a,C}	10.5 ^{a,B}	10.4 ^{a,B}
RC	11.1	11.1	10.1	10.5	10.6	10.7 ^{v,W}	9.6	9.6	8.8	8.7	8.7	9.1 ^{w,W}	10.4 ^{b,A}	10.4 ^{a,A}	9.5 ^{b,B}	9.6 ^{b,B}	9.6 ^{b,B}
LC	10.6	10.5	10.4	10.2	10.4	10.4 ^{v,X}	8.7	8.8	8.7	8.2	8.1	8.5 ^{w,X}	9.6 ^{c,A}	9.7 ^{b,A}	9.6 ^{b,A}	9.2 ^{c,B}	9.2 ^{c,B}
BT	9.9	10.2	9.7	10.1	10.4	10.1 ^{v,Y}	8.2	8.3	7.8	7.7	8.1	8.0 ^{w,Y}	9.1 ^{d,AB}	9.3 ^{c,A}	8.7 ^{c,C}	8.9 ^{d,BC}	9.3 ^{c,A}
Mean	10.8 ^{g,G}	10.7 ^{g,G}	10.2 ^{g,I}	10.5 ^{g,H}	10.7 ^{g,G}	10.6	9.2 ^{h,G}	9.1 ^{h,G}	8.7 ^{h,H}	8.6 ^{h,H}	8.6 ^{h,H}	8.8					

^{a,b,c,d,e} LSMeans are significantly different between species within cutting date at $P<0.05$; $SE_{SC} = 0.12$; $SE_{SG} = 0.08$; $SE_{RG} = 0.08$

^{A,B,C,D} LSMeans are significantly different between cutting dates within species at $P<0.05$; $SE_{SC} = 0.12$; $SE_{SG} = 0.08$; $SE_{RG} = 0.08$

^{g,h} LSMeans are significantly different methods within cutting date at $P<0.05$; $SE_{SC} = 0.07$; $SE_{SG} = 0.04$; $SE_{RG} = 0.05$

^{G,H,I,J} LSMeans are significantly different between cutting date within method at $P<0.05$; $SE_{SC} = 0.07$; $SE_{SG} = 0.04$; $SE_{RG} = 0.05$

^{v,w} LSMeans are significantly different between methods within species at $P<0.05$; $SE_{SC} = 0.09$; $SE_{SG} = 0.04$; $SE_{RG} = 0.04$

^{V,W,X,Y,Z} LSMeans are significantly different between species within method at $P<0.05$; $SE_{SC} = 0.09$; $SE_{SG} = 0.04$; $SE_{RG} = 0.04$

ME contents of forage legumes from different defoliation systems from the study site Germany were always significantly higher (P<0.05) when estimated based on CM than on TT (Table 8). Within the dataset from the SC system from Germany, the ranking of the species as means over cuts was in general similar to the described SC system data including both study sites. Here, the additional legume species kura clover (KC) differed, as MECM was equal to WC, and METT ranged between WC and RC, significantly differing from both species. The range of the largest and smallest mean was numerically larger for METT than MECM, as is valid for all defoliation systems. Estimated as MECM, energy contents as means over cuts declined from the 1st to the 3rd cut, whereas METT was equal in the 2nd and 3rd cut.

Data of the simulated grazing system (SG) showed consistency in the ranking of species within the methods as means over cuts, with all species differing significantly from each other, giving ME highest for WC > KC > RC > LC > BT. This is similar for the rotational grazing system (RG), where KC was not included. Deviations between the respective methods

appeared in ME of the cuts as means over species. Within a comparative consideration of the MECM and METT for each cut and species in the SG and the RG system, the course of the ME values during the vegetation period showed a good conformance within the first cutting dates, whereas larger deviations could be observed in the 4th and 5th cut. In the SG system, MECM of red clover, lucerne and birdsfoot trefoil were high in the forage of the 4th and 5th cut, whereas METT was similar or lower.

Figure 6 shows the relationship between the ME of all samples included in the evaluation calculated as mean values of the respective field replicates (n=149). The generally smaller METT values observed were reflected in the regression equation, the intercept (-1.35) differing significantly from zero ($P<0.05$), whereas the slope did not differ from unity. The large variation around the regression line resulted in a relatively poor correlation coefficient ($R^2=0.63$)

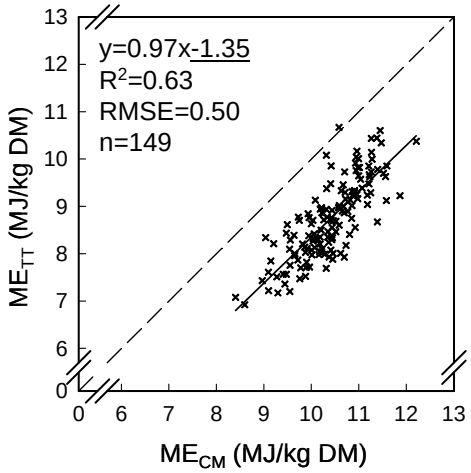

Figure 6: Relationship between ME of several forage legumes estimated by NIRS based on Cellulase method (ME_{CM}) respectively Tilley and Terry (ME_{TT}). Graphs include all data of both study sites and years (if available) as means over field replicates. Bisector shown as dashed line. Underlined regression parameters differ significantly from unity (slope)

Separation by defoliation systems (Figure 7) did not improve the results compared to the whole dataset; the intercepts for the regression lines were significantly different from zero within the SG and the RG datasets, whereas the slopes did not differ from unity.

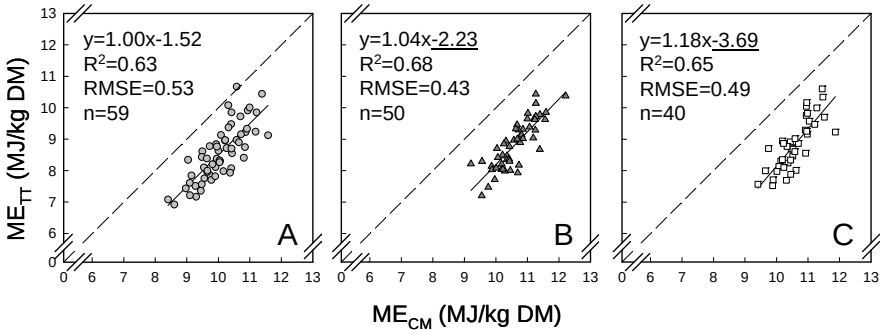

Figure 7: Relationship between ME of several forage legumes estimated by NIRS based on Cellulase method (ME_{CM}) and Tilley and Terry (ME_{TT}). Graphs include data from both study sites and years as means over field replicates, grouped by defoliation systems: **A** silage cutting; **B** simulated grazing; **C** rotational grazing. Bisector shown as dashed line. Underlined regression parameters differ significantly from unity (slope) respective zero (intercept)

In regard to the main factor species, separate regression analyses were performed for each legume species (Figure 8). Four of five species, i.e. white clover, red clover, lucerne, and birdsfoot trefoil, showed a slope significant smaller than unity, whereas no significant

difference of the intercepts from zero could be observed for the species. Although computing comparable value for slope as white clover, a significant deviation was not observed for kura clover, which may be related to the narrow range of the ME values. Thus, for all species the range of the means was numerically larger for METT than MECM.

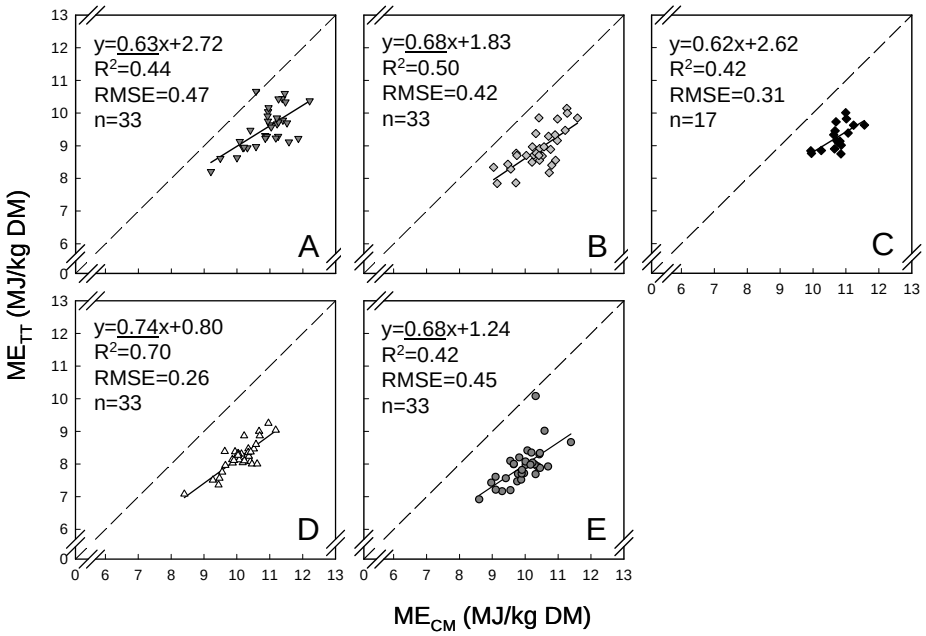

Figure 8: Relationship between ME of several forage legumes estimated by NIRS based on Cellulase method (ME_{CM}) respectively Tilley and Terry (ME_{TT}). Graphs include data from both study sites and years (if available) as means over field replicates, with samples grouped by species: **A** white clover; **B** red clover; **C** kura clover; **D** lucerne; **E** birdsfoot trefoil. Bisector shown as dashed line. Underlined regression parameters differ significantly from unity (slope) respective zero (intercept)

The regression analysis considering cuts within systems (Figure 9) did not achieve further improvement. Some significant effects could be proved; however, these datasets included a broader range of ME and gained relative good correlation coefficients, whereas for intercepts and slopes of other regression lines, no differences were observed due to a restricted number of data pairs. Overall, the intercepts tended to be numerically negative, which indicated a larger deviation of ME values for samples with low energy content, whereas for the slopes no consistent trend was observed.

3.2.4 Discussion

As shown by the mixed model and regression analyses of all data included, the determination of metabolisable energy (ME) of forage legumes clearly differed depending on the underlying in vitro method used, with systematic higher values for the pepsin-cellulase method (CM).
Reasons can be found in the general limitations given within the methods and the respective evaluation methods. Due to the use of a correction step in the calculation of the OMD values with TT, laboratory results are adapted to the level of the in vivo values of the standard samples after each measurement, whereas the equation for CDOM determination was validated on in vivo estimations (VDLUFA, 1993). De Boever et al. (1986) reported that CDOM for high quality forages and concentrates was higher than in vivo values, whereas it was lower for forages with in vivo DOM less than about 70%, with differences increasing with declining quality. This does not apply to the standard samples used in the present experiment, as most in vivo DOM values were less than 70%.
Apart from this, the higher MECM values may be a result of a general overestimation by the CM method. Further, a larger variation of the values obtained from the TT procedure was reflected in the larger variation of the results of the standard sample analysis, indicating the disadvantages of a method using natural inoculum.

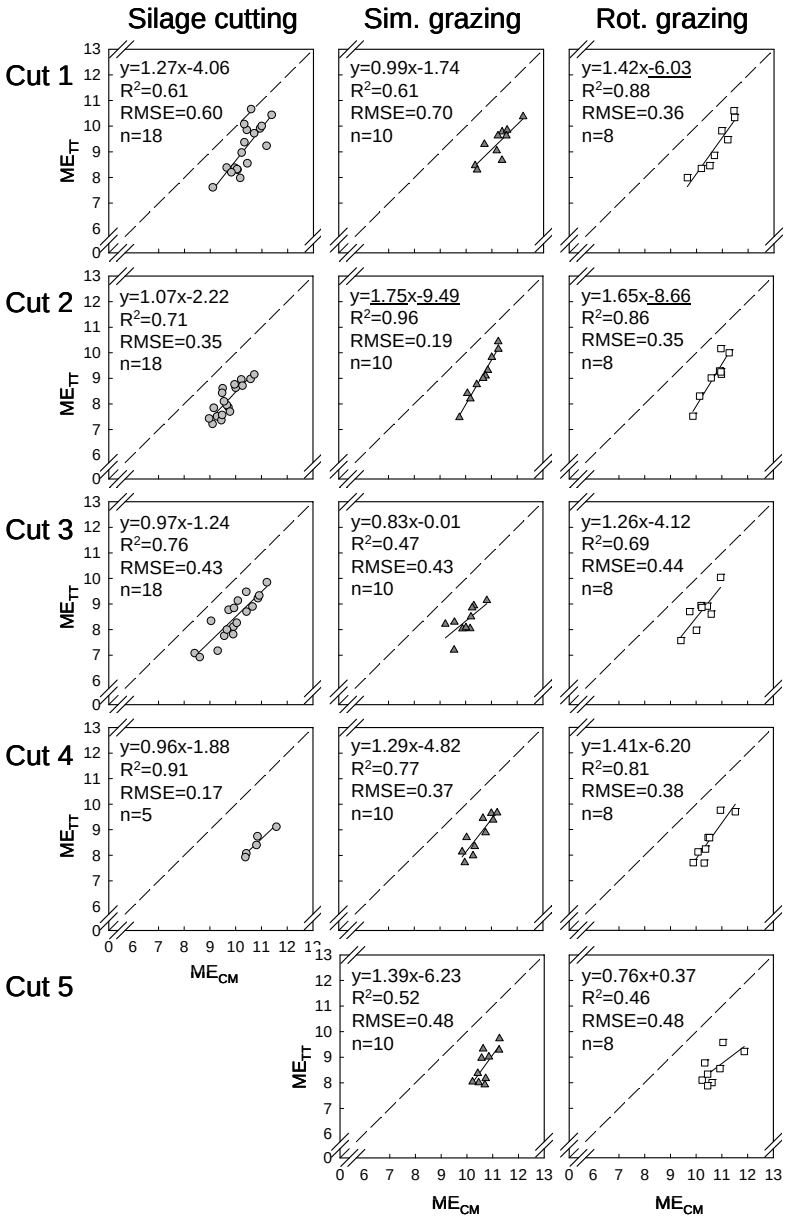

Figure 9: Relationship between ME of several forage legumes estimated by NIRS based on Cellulase method (ME_{CM}) respective Tilley and Terry (ME_{TT}). Graphs include data from both study sites and years (if available) as means over field replicates, grouped by defoliation systems and cuts. Bisector shown as dashed line. Underlined regression parameters differ significantly from unity (slope) respective zero (intercept).

Whereas factors, as variation in the activity of rumen microbes between donor animals, sampling days, and finally analysis runs (e.g. Weiss, 1994; Stern et al., 1997) could be prevented due to appropriate practices and the mathematical correction, another critical point is possibly the diet of the donor animals. The influence of the diet of the donor animal on the inoculum activity is controversially discussed in the literature (e.g. reviewed by Nefzaoui and Vanbelle, 1985). As the basal diet can be a major source of variation for in vitro OMD data, it was frequently indicated that donor animals should be fed on diets that are similar in quality characteristics to feed samples which will be analysed using the rumen liquor to allow an adaptation and sufficient growth of a mixed microflora (Horton et al., 1980; Jung and Varel, 1988; Ayres, 1991; Holden, 1999; Iantcheva et al., 1999; Mabjeesh et al., 2000). Donor animals in this study were fed on green forage from mixed swards from Austria, which contained grass as well as legumes and herbs allowing the analysis of all samples in routine

analyses. However, legume content was about 10-15% in the standards and mainly determined by white clover and probably low proportions of red clover and lucerne, whereas birdsfoot trefoil and kura clover were absent. In consequence, determination of OMD showed lower variation using CM. As found in other studies, the in vitro CDOM values generally agree rather well with in vivo values, regardless of the feed type analysed (De Boever et al., 1988), whereas Weiss (1994) stated that the estimation of forage digestibility with enzymatic methods generally generates larger error than that for concentrates.

The correlation coefficient for TT decreased even more for the NIRS estimation of METT, whereas it gained for MECM at a similar level as for the CDOM, indicating sources of error within the calculation of the METT values. The regression of OMD values and ME contents obtained from the DLG tables (DLG, 1997), as done in this study separately for the first cut and regrowths, is in general a suitable method to derive energy values, since these tables are based on a large number of in vivo digestibility experiments and generally result in a good rating. Though, deviations may be caused by regression with ME values for green fodder without consideration of legume species. Concerning the samples of the 5-cut systems from Germany, a disregard of the defoliation frequency may have caused the even higher deviations of the intercepts which have been found in the regression analyses of the samples separated by management systems (Figure 2.3). The plants were harvested at an earlier vegetation stage and thus have commonly higher ME values, which might have generated misleading results due to underestimation of METT. That may be a general source of error; though, on the one hand a regression separated by legume species with the respective values derived from DLG tables gained no increase in the in vitro correlation coefficient for OMD (R. Resch, personal communication, 2007), on the other hand some species are not included in the guidelines, as no data are given for birdsfoot trefoil or kura clover. It has to be mentioned that values in the DLG tables were derived from feed trials with forages grown under German conditions, limiting the transferability to samples from other origins. Gosselink et al. (2004) found low prediction errors (5.0%) for OMD of a set of legume and grass samples by TT compared to in vivo OMD, and inclusion of chemical components did not improve the predictions. Jones and Theodorou (2000) pointed out that the usefulness of CM to measure digestibility in forage evaluation will ultimately depend on the reliability and consistency of the predictive equations derived for in vivo digestibility. Prediction of in vivo digestibility based on the CM and the TT method was compared for grasses, lucerne, red clover and sainfoin (Terry et al., 1978), resulting in comparable accuracy for grasses, but enzyme solubility was less accurate for predicting the digestibility of legumes. Accurate prediction equations are even more important when further calculations are drawn from digestibility values, as discussed for the ME contents. For the CM, a regression equation was developed for ME estimation separately for legumes by Weissbach et al. (1996). This equation is based on 20 in vivo digestibility trials including lucerne and red clover, preserved as silage, hay, and oven-dried forage, in all covering a range of 29 to 77% OMD.

Enzyme-based predictions of in vivo digestibility and energy value can vary with forage species, population and harvest season (Barber et al., 1990; Givens et al., 1995). Differences between growths were proved as significant in in vivo digestibility equations for grasses for enzyme as well as rumen inoculum methods (Jones and Theodorou, 2000). In birdsfoot trefoil of two morphological forms, in vivo digestibility and estimated ME showed only minor decline during maturity, and differences between morphological forms were only significant for very mature plants (Ramírez-Restrepo et al., 2006). However, for other legumes, as lucerne, red clover, or white clover, morphological differences during growing season are larger and thus have a stronger influence on digestibility and ME content due to an altered leaf:stem ratio and changes in nutritional quality of each fraction with maturity (Nordkvist

and Åman, 1986; Wilman and Rezvani Moghaddam, 1998; Ammar et al., 1999). In white clover, leaflets had a lower NDF and lignin content on DM basis than the stolons and petioles and were less digestible than the latter (Wilman and Rezvani Moghaddam, 1998). Lignin content is the variable most closely linked to in vivo digestibility. Species differ in the relationship of pepsin-cellulase solubility to in vivo digestibility (Terry et al., 1978), probably due to the fact that enzyme methods solubilise less of the cell wall than in vivo digestion and are more influenced by the degree of lignification (Jones and Theodorou, 2000). As environmental factors influence content and degree of lignification of the cell wall, the extent to which predictive equations derived from enzyme solubility apply to different environmental and other conditions. As it is known, red clover contains the enzyme polyphenol oxidase in the leaves, which produces quinones and in this way may cause a complexation of proteins. This may have a stronger impact on the rumen microbes of the TT method, whereas the effect may be ignored in the CM due to the unsuitable pH value in the pepsin step. A similar explanation can be drawn for the effect of condensed tannins, contained in birdsfoot trefoil, which might act on rumen microbes, but not during the pepsin-cellulase procedure of CM. Tannins are generally regarded as inhibitory to microorganism growth and activity, the extend dependent on their concentration. For ruminal bacteria, differences in their tolerance of tannins were found in in vitro tests (McSweeney et al., 2001). As these studies usually excluded media constituents such as protein that would complex with free tannin, the interaction between tannins and microorganisms in the rumen will probably differ (McSweeney et al., 2001), and likewise in in vitro analyses of forages with rumen fluid (Makkar, 2005). Reviewed by Aufrère and Guérin (1996), prediction of OMD by enzymatic methods is in general accurate, except for forages containing tannins. Reasons suggested for this is on the one hand the pH value during the pepsin treatment (pH of approx. 3) differing from the pH value in the rumen, thus tannin-protein complexes could dissociate (Martínez and Moyano, 2003) and lead to an overestimation of OMD for tannin-containing forages compared to in vivo digestibility.

On the other hand, for some kinds of tannins an inhibiting effect on the enzyme activity (e.g. cellulases) is assumed (Aufrère and Guérin, 1996). For forages containing tannins, adaptations in the determination of fiber and protein digestibility were provided (Robbins et al., 1987a,b; Hanley et al., 1992). Similar effects might occur under appropriate conditions in forage plants containing polyphenol oxidase activity, as the produced quinones may act in some cases comparable to the effects of condensed tannins.

The relationships between the results obtained by the estimated METT and MECM contents are not consistent, as shown when determined separately for legume species or cuts (Figures 2.4 and 2.5). Both secondary plant components, polyphenol oxidase (Fothergill and Rees, 2006; Eickler et al., 2007; Winters et al., 2008) and condensed tannins (Cassida et al., 2000; Gebrehiwot et al., 2002; Julier et al., 2003), vary in their contents during growing season and may contribute to differences in the quality evaluations due to their different effects on the in vitro methods. Thus, for a scientific approach, the values gained from the two methods of pure legume samples may be misleading to some extent and partially explain the observed cut × species interactions. However, it may be discussed if the resulting differences in the estimation of OMD and ME based on the two methods are a result of single forage analysis (Friedel et al., 1999). The authors pointed out that with cellulase techniques, comparable values for the digestibility can be derived, as would be the case in standardised digestibility trials. However, in mixed diets the tannin content of single feeds is likely to be diluted to have any measurable effect on digestibility in the rumen. In fact, the ranking of the ME contents of forage legume species as means over cuts are in general comparable between both methods (Table 2.5 and 2.6), with exception of kura clover of the simulated grazing system in

Germany (Table 2.6), which was estimated with the same level as white clover by the CM, whereas by TT contents of ME were significantly lower than the ME of white clover. Thus, for a general comparison between the species in spite of in vivo values, both methods seem to be suitable. It may be suggested that the TT procedure may lead to false estimation if microbial activity is influenced by secondary compounds in forages like condensed tannins and polyphenol oxidase activity, resembling estimation of OMD of unadapted ruminal microflora. In contrast, the CM is not sensitive to the secondary compounds and seems to be more suitable for the estimation of OMD of already adapted animals. It was suggested that in vivo OMD is the best reference method for developing NIR equations for OMD (Murray, 1993; Deaville and Flinn, 2000). Berglund et al. (1990) intended that NIR data should be calibrated using in vivo material, and the predictions should be compared with the accuracy of other indirect methods in predicting metabolism trial results to obtain an accurate evaluation of the NIR approach. The main difficulty consists of the requirement of in vivo data obtained from a large number of digestion trials, conducted under standardised conditions, as it was the case in the present experiment. Moreover, in regard to the renewed interest in the nutritional value and quality of forage legumes in general, and even more concerning species with secondary plant components with their potential benefits and limitations, their influence on the ruminant nutrition has to be investigated in detail in in vivo studies to evaluate their potential in animal nutrition. On this basis, appropriate laboratory methods can be identified and respective equations for estimation or calibration adapted to establish reliable and robust prediction tools to be integrated in the routine analysis as well as for scientific approaches.

3.2.5 Conclusions

From a strict point of view, the estimation of ME contents of forage legumes is far more succeeding based on the pepsin-cellulase method due to the higher precision and correlation to in vitro values. The Tilley and Terry procedure may thus provide a reasonable higher accuracy due to the possible interaction of the rumen microbes with plant components, as it is the case for in vivo measurements. To meet the criteria for standardisation, the use of the TT method for application on a large sample set and time scale is limited. Moreover, our results showed a systematic deviation between the tested in vitro methods and the respective ME calculations for forage legume species. For the improvement of the laboratory methods as well as to derive robust NIR calibrations, more in vivo studies have to be conducted under standardised conditions, providing reference samples as benchmarks in the routine analysis as well as in a scientific context.

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4. Further activities within COST 852 respectively concerning project BAL 2729

4.1 publications

EICKLER, B. M. GIERUS, E.M. PÖTSCH, R. RESCH, F. TAUBE (2007): Schätzung der Energiegehalte von Futterleguminosen mittels zweier in vitro-Methoden. 119. VDLUFA-Kongress, Kurzfassungen der Referate, S. 66.

EICKLER, B., GIERUS, M., PÖTSCH, E.M., RESCH, R. und F. TAUBE (2007): Vergleich zweier in vitro-Methoden zur Schätzung der Energiegehalte von Futterleguminosen. Tagungsband der 51. Jahrestagung der AGGF, Göttingen, 105-108

EICKLER, B., GIERUS, M., PÖTSCH, E.M., RESCH, R. und F. TAUBE (2007): Bestimmung des energetischen Leistungsvermögens von Futterleguminosen - Vergleich zweier in vitro-Methoden. Mitt. Ges. Pflanzenbauwiss. 19, 156-157

EICKLER, B., M. GIERUS, E. M. POETSCH, F. TAUBE (2005): Influence of secondary plant components on protein quality and digestibility of forage legumes. COST Action 852 Working Group III Meeting in Grado (Italy).

GIERUS, M., VAN DORLAND, A., ELGERSMA, A., KREUZER, M., LOGES, R., PÖTSCH, E.M., SMIT, H., STEINSHAM, H., TAUBE, F. and M. WACHENDORF (2007): Influence of contrasting environments on forage quality of ryegrass and four legumes growing in binary swards. Grassland Science in Europe, Volume 12, "Permanent and temporary grassland – plant, environment and economy", 162-165

HELGADÓTTIR Á. and E.M. PÖTSCH (2007): Quality legume-based forage systems for contrasting environments. Proceedings of the Final Meeting edit by Áslaug Helgadóttir and Erich M. Poetsch, ISBN: 978-3-902559-07-4, pp 242

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PÖTSCH, E.M., B. EICKLER, M. GIERUS and R. RESCH (2005): Productivity of legume/grass mixtures in mountainous regions of Austria. Presentation of first results from the common COST 852-experiment. Workshop at Grado, Italy

Wachendorf, M., Kreuzer, M., Collins, R.P., de Faccio Carvalho, P.C., Dewhurst, R.J., Elgersma, A., GIERUS, M., HÖGLIND, M., HUYGHE, C., LOGES, R., PARENTE, G., PÖTSCH, E., SÜDEKUM, K.H., STEINSHAMN, H., TAUBE, F., VAN DORLAND, H.A., VENERUS, S., WETTSTEIN, H.-R., 2004. Utilisation of forage legumes: plant and animal science linked in the common protocol of COST Action 852 – Working Group 3. In Lüscher, A., Jeangros, B., Kessler, W., Huguenin, O., Lobsiger, M., Millar, N., Suter, D. (eds) Land use systems in grassland dominated regions. Book of abstracts, EGF 2004, Luzern, Switzerland, pp.93-94.

4.2 presentations/lectures

PÖTSCH, E.M.: Activities on legumes at the Federal Research Institute for Agriculture in Alpine Regions, COST 852 meeting, Centre Tecnologic Forestal de Catalunya, Solsona, Spain, 23.2.2002

PÖTSCH, E.M.: Floristic diversity and forage quality of mountainous pastures in Austria, 13th Meeting of the FAO-CIHEAM Mountain Pastures Network, Udine, Italy, 15.09.2005

PÖTSCH, E.M.: Productivity of legume/grass mixtures in mountainous regions of Austria, COST 852 Meeting and Workshop, Grado, Italy, 10.11.2005

PÖTSCH, E.M.: In-vitro digestibility and energy concentration of different legumes – results from the COST 852 experiment in Austria, Final Meeting of COST 852 "Quality-based forage systems for contrasting environments", HBLFA Raumberg-Gumpenstein, Austria, 01.09.2006

PÖTSCH, E.M.: Low input farming systems & livestock production – grassland and dairy farming in Austria, Summer University, Ranco, Italy, 03.07.2007

PÖTSCH, E.M.: Semi-natural grassland as a source of biodiversity. 15th Meeting of FAO-CIHEAM Mountain Pastures Network, Les Diablerets, Switzerland, 07.10.2009

4.3 scientific missions and supervision

EICKLER, B.: Scientific long term mission "Legume-Based Forage Systems for Contrasting Environments" within Marie Curie Training site MounTrain at AREC Raumberg-Gumpenstein, Austria, May – December 2005

RESCH, R.: Short term scientific mission "Polyphenoloxidase determination methodology in red clover samples" at CA – University at Kiel, Germany, 30.10.2006 -04.11.2006