



## Evaluation of Chemical Composition and *In vitro* Digestibility of Appennine Pasture Plants Using Yak (*Bos grunniens*) Rumen Fluid or Faecal Extract as Inoculum Source\*

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**ABSTRACT** : Pastures of the Apennines of Central Italy contribute to feed resources of high altitude (above 1,300 m sea level) grazing systems. The objective of this study was to evaluate the effectiveness of faecal extract from the yak (*Bos grunniens*) as an alternative microbial inoculum to rumen fluid for estimation of digestibility of several forage species. Forage samples produced at high altitude were tested in this study: four legumes (*Lathyrus sativus* L., *Lotus corniculatus* L., *Onobrychis viciaefolia* L. and *Trifolium pratense* L.), three forbs (*Achillea millefolium* L., *Potentilla reptans* L. and *Teucrium flavum* L.) and one grass (*Brachipodium pinnatum* L.) were incubated with yak rumen fluid or faecal extract. A large variability in chemical composition was observed among the species collected. Rumen liquor and faecal samples were collected from adult healthy yak. The Daisy<sup>II</sup> incubator was used to evaluate the nutrient digestibility of forages using rumen liquor as control and faecal extract as alternative microbial inoculum sources. Filter bags containing samples of browse species were added to the four digestion vessels along with their respective inoculum and then incubated for 48 h and dry matter (DM), organic matter (OM), crude protein (CP), neutral and detergent fiber (NDF) digestibility was determined. There was a significant relationship between estimates, indicating that faecal liquor has the potential to be used instead of rumen fluid for estimation of *in vitro* digestibility of plants. It is concluded that the Daisy<sup>II</sup> incubator results are appropriate for the determination of *in vitro* digestibility of nutrients using faecal liquor to define the potential for adaptation of yak to new pastures. (**Key Words** : Yak, Pasture Quality, Faecal Extract, *In vitro* Digestibility)

### INTRODUCTION

Yak (*Bos grunniens*), the multipurpose bovid which provides milk, meat, wool and much-needed pack capability on precipitous slopes, belongs to the sub-family Bovine of the family *Bovidae*. The total world population of yak is about 15 million which are found in China, Mongolia, Russia, Pakistan, India, and Nepal. A drastic decline in yak population all over the world has become a concern to development authorities and to animal scientists because

this animal caters to the needs of the highlanders whose economy largely depends on yak husbandry, besides sheep and goat rearing (Sarkar et al., 2006).

The yak, like other grazing ruminants, has a highly developed and specialized mode of digestion that has evolved to maximize the utilization of carbohydrates from cellulose and thus allows better access to energy in the form of fibrous feeds than in non-ruminant herbivores (Dong et al., 2006).

Chemical composition of pasture vegetation is crucial, particularly in combination with *in vitro* digestibility, to evaluate the nutritive value of browse species which are not known previously (Laudadio et al., 2009). Consequently, a detailed survey of browse species is important to identify the better shrub species for ruminants, in terms of nutrient content and digestibility. *In vitro* digestion techniques using rumen liquor as a microbial inoculum (Tilley and Terry, 1963) have proved useful in assessing the relative digestibility of many feeds (Minson, 1990).

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The necessity for fistulated animals to provide this inoculum raises a number of practical problems, e.g. surgical facilities, constant care to avoid infections and costs associated with the long-term maintenance of these animals (Mauricio et al., 2001). Several studies, reviewed by Omed et al. (2000), have demonstrated faeces to have high potential as an alternative inoculum for *in vitro* digestibility techniques. The successful use of a liquid suspension of faeces has been reported from sheep (Váradyová et al., 2005), cattle (Holden, 1999; Mabjeesh et al., 2000) and recently from horses (Lattimer et al., 2007; Murray et al., 2008) to estimate digestibility of a range of feeds.

The search for better labor efficiency has led to the development of the Daisy<sup>II</sup> apparatus (ANKOM<sup>®</sup> Technology Corp., Fairport, NY), which allows simultaneous incubation of different feedstuffs in sealed polyester bags in the same incubation vessel. Results previously showed that the use of rumen fluid or faecal liquor inoculum (Lattimer et al., 2007; Tufarelli et al., 2010) with a closed-system fermentation apparatus (Daisy<sup>II</sup> Incubator) yielded valid *in vitro* estimates of dry matter and fibre digestibility of forages and grains. Furthermore, this technique allows the estimation of *in vitro* digestibility of a large number of samples simultaneously, in addition to recovery of the residue for the final prediction of *in vitro* digestibility of feeds.

Since several factors contribute to differentiation of pastures in terms of nutritive value, which have to be accurately estimated to determine the most appropriate grazing strategy, the objective of this study was to evaluate the different sources of rumen inocula from yak for effectiveness in assessing nutrient digestibilities of several high altitude forages.

## MATERIALS AND METHODS

### Study area and plant sample collection

The study area consisted of a 500 ha grazing area located in the Central Apennines of Italy (Roiano, Teramo province) at an altitude of between 1,300 and 1,500 m (above sea level). Pastures were subjected to a moderate grazing pressure during spring-summer. Local climate is characterized by cold winters and temperate summers, with a mean annual precipitation of 850 mm occurring with a maximum in spring and autumn and a minimum in August. Total rainfall during the sampling period was approximately 260 mm, with an average temperature of 15°C. The soil, laying on a calcareous parent rock, has a sandy clay texture and is moderately acid.

Plant composition of the pastures was assessed monthly. The assessment of each browse species and the dry matter

produced was sampled at monthly intervals from May to July of 2009 using three plots obtained from three series of parcels every month. Thus, a pasture area of 5 m<sup>2</sup> was sampled on each of three occasions. The sampling criterion was that samples would reflect the nutritive conditions of the species as they were found by the grazing herbivores in the study area.

Eight Apennine browse species were investigated as follows: four legumes (*Lathyrus sativus* L., *Lotus corniculatus* L., *Onobrychis viciaefolia* L. and *Trifolium pratense* L.), three forbs (*Achillea millefolium* L., *Potentilla reptans* L. and *Teucrium flavum* L.) and one grass (*Brachipodium pinnatum* L.). Approximately 100 g of fresh sample was taken before grazing by clipping at the browsing height of yaks: upper half of unfertile leaves (tiller) for grass, total stem with their leaves (10 cm high) for forbs and current growth (5 cm) for the legumes.

### Chemical analysis

Samples were ground in a hammer mill passing a 1-mm screen and analysed in duplicate for dry matter (DM), ash, crude protein (CP, Kjeldahl N×6.25), crude fibre (CF) and ether extract (EE) (with previous hydrolysis in compound samples) according to the procedures outlined by the AOAC (2000). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined with the ANKOM fibre analyser according to Van Soest et al. (1991), and were corrected for residual acid-insoluble ash. Sodium sulphite, but not  $\alpha$ -amylase, was added to the solution for the NDF determination. Acid detergent lignin (ADL) was determined by the method of Van Soest and Robertson (1985).

For mineral estimation, 1 g of dried sample was ignited in a muffle furnace at 550°C for 4 h. The ash was dissolved in hydrochloric acid and filtered through filter paper, and the final volume was made to 250 ml with Milli-Q water (Millipore Corporation, Bedford, MA, USA). All minerals (*i.e.*, Ca, P, Mg, Na, K, Cu, Fe, Mn, Zn) were estimated using an atomic absorption spectrophotometer. A specific lamp was used for each mineral and the atomic absorption spectrophotometer was calibrated with various concentrations of mineral standards. The standards and samples were positioned and injected using the furnace auto-sampler. To determine the content of Ca and Mg, processed samples were further diluted with lanthanum chloride to mask interferences of other minerals.

### *In vitro* digestibility determination

In order to study *in vitro* digestibility parameters, rumen fluid was obtained from the rumen of four mature healthy yaks (*Bos grunniens*), reared in the same study area and fed diets sufficient for their maintenance allowance (NRC, 2001), using an oesophageal tube under mild vacuum from

the reticulum near the reticulo-omasal orifice. Faeces were collected from the same animals, consuming the same diet, by collecting fresh faeces directly from the rectum using palpation sleeves. Samples of ruminal contents (filtered through eight layers of gauze cloth) and faeces were collected in thermos flasks and taken immediately to the laboratory where they were strained through various layers of cheesecloth and kept at 39°C under a CO<sub>2</sub> atmosphere until use (within approximately 20 min). The faecal inoculum was prepared by homogenizing 40 g of faeces with 360 ml of warm, distilled water for 2 min under CO<sub>2</sub> and then filtering through double-layered cheese cloth directly into the pre-warmed digestion vessels.

*In vitro* fermentation was conducted for 48 h using the Daisy<sup>II</sup> incubator system. The complete unit consisted of four incubation vessels with a capacity of 2 L each. Each vessel contained 1.6 L of buffer solution, 400 ml of either rumen liquor or faecal extract as the inoculum, and 22 nylon bags. The buffer solution consisted of 1.33 L buffer A (KH<sub>2</sub>PO<sub>4</sub>, 10.0 g/L; MgSO<sub>4</sub>7H<sub>2</sub>O, 0.5 g/L; NaCl, 0.5 g/L; CaCl<sub>2</sub>2H<sub>2</sub>O, 0.1 g/L; and urea, 0.5 g/L) and 266 ml of buffer B (Na<sub>2</sub>CO<sub>3</sub>, 15.0 g/L and Na<sub>2</sub>S7H<sub>2</sub>O, 1.0 g/L), mixed in each digestion vessel and the pH was adjusted to 6.8. Each sample was digested in duplicate for each source of inoculum. Nylon filter bags (ANKOM F57, ANKOM Tech., Fairport, NY) were rinsed in acetone and allowed to air dry before drying at 100°C for 24 h, after which dry bag weight was recorded. For each browse plant species, 0.25 g (Lattimer et al., 2007) of ground sample was added to a nylon bag which was then dried at 105°C for 24 h, after which dry sample plus bag weight was recorded. For each analyzed parameter *in vitro* digestibility (IVD) was calculated as:  $IVD (\%) = (P_{BD} - P_{AD}) / P_{BD} \times 100$ , where P<sub>BD</sub> is the percentage of parameter in the samples before the digestion and P<sub>AD</sub> the percentage of the parameter after the

digestion. Duplicate nylon bags for each species were randomly allocated to one of the four digestion vessels, and therefore to one of the two inoculum treatments.

### Statistical analysis

The effect of treatment was compared by analysis of variance procedures and the contrast of means by Tukey's test, using Proc Mixed of SAS statistical software (SAS, 2000). In the statistical model, the incubation run was considered as a blocking factor, and the source of inoculum (yak rumen fluid vs. faecal extract) as the treatment factor.

## RESULTS

### Pasture chemical composition

Chemical and nutrient composition of browse plants collected during the experimental period are reported in Table 1. As expected, the analysis of plot cover and chemical measurements showed clear differences between species. The DM content of analyzed plants varied between 198 g/kg for *Achillea millefolium* and 235 g/kg for *Teucrium flavum*. All the samples had similar organic matter (OM) content. The CP content of pasture plants ranged from 94 g/kg for *Potentilla reptans* and 210 g/kg for *Trifolium pratense*. The crude fibre had an overall mean value of 42 g/kg and ranged from 31-53 g/kg for *Trifolium pratense* and *Potentilla reptans*. The mean value for NDF content varied between 315 g/kg in *Lotus corniculatus* to 761 g/kg for *Brachipodium pinnatum*. The ADF ranged from 211 g/kg for *Lotus corniculatus* to 646 g/kg in *Brachipodium pinnatum*, with a pasture mean value of 291 g/kg. Data obtained on ether extract content in browse plants showed a low mean value of 13 g/kg. The highest and lowest values of lignin(sa) were 58 g/kg in *Achillea millefolium* and 36 g/kg in *Teucrium flavum*. The crude ash

**Table 1.** Chemical composition (g/kg dry matter) of some Appennine browse species

Plant species	Dry matter	Organic matter	Crude protein	Crude fibre	NDF	ADF	ADL	Ether extract	Ash
<b>Legumes</b>									
<i>Lathyrus sativus</i>	214	887	137	35	401	312	42	11	113
<i>Lotus corniculatus</i>	231	879	122	39	315	211	55	13	121
<i>Onobrychis viciaefolia</i>	207	876	141	44	412	307	49	9	124
<i>Trifolium pratense</i>	215	849	210	31	397	279	47	10	151
<b>Forbs</b>									
<i>Achillea millefolium</i>	198	867	151	49	367	255	58	13	133
<i>Potentilla reptans</i>	203	852	94	53	612	501	40	14	148
<i>Teucrium flavum</i>	235	860	129	51	554	422	36	15	140
<b>Grasses</b>									
<i>Brachipodium pinnatum</i>	209	871	104	36	761	646	49	16	129
SEM	17.1	9.7	12.3	6.2	41.4	43.3	5.9	3.3	9.8

NDF = Neutral-detergent fibre; ADF = Acid-detergent fibre; ADL = Acid-detergent lignin.

SEM = Standard error of the means.

**Table 2.** Composition of macro- and micro-minerals of different Appennine browse species

Plant species	Macrominerals (g/kg)*					Microminerals (mg/kg)*			
	Ca	P	Mg	Na	K	Fe	Cu	Mn	Zn
Legumes									
<i>Lathyrus sativus</i>	35.7	1.1	0.8	0.6	9.7	522	33	171	95
<i>Lotus corniculatus</i>	44.8	0.9	0.9	0.5	11.3	487	26	147	101
<i>Onobrychis viciaefolia</i>	37.8	0.8	1.1	0.4	10.1	491	25	166	103
<i>Trifolium pratense</i>	26.3	1.3	1.4	0.6	17.2	592	31	151	91
Forbs									
<i>Achillea millefolium</i>	19.2	1.5	2.1	0.5	25.5	277	18	94	93
<i>Potentilla reptans</i>	23.9	1.4	1.2	0.6	13.6	305	14	101	95
<i>Teucrium flavum</i>	15.3	1.4	0.9	0.5	10.1	288	19	103	102
Grasses									
<i>Brachipodium pinnatum</i>	10.9	1.2	0.9	0.3	10.7	391	10	68	79
SEM	5.1	0.4	0.2	0.2	1.3	21.2	2.7	13.3	4.4

\* Values on dry matter basis. SEM = Standard error of the means.

level of vegetation showed a range of variability between 113 g/kg for *Lathyrus sativus* and 151 g/kg for *Trifolium pratense*.

Mineral composition of the eight browse species is presented in Table 2. In the majority of plants, macro-elements Ca, P, Mg, Na and K were relatively high, while the micro-elements (Cu, Fe, Mn and Zn) contents were low. In particular, Ca contents (g/kg DM) ranged from 10.9 and 44.8 for *Brachipodium pinnatum* and *Lotus corniculatus*, respectively. Moreover, P and Na concentrations in all selected species were similar (1.3 and 0.5 g/kg DM, respectively). Similar values (overall 1.1 g/kg DM) were evident also for Mg, except in *Achillea millefolium* with 2.1 g Mg/kg DM. A large range of variation was found in K values (9.7 to 25.5 g/kg, in *Lathyrus sativus* and *Achillea millefolium*, respectively).

Micro-mineral concentrations (expressed in mg/kg DM) registered high levels of Fe, between 277 and 592 in *Achillea millefolium* and *Trifolium pratense*, respectively. Cu content ranged from 10 to 33 mg/kg DM in *Brachipodium pinnatum* and *Lathyrus sativus*, respectively. Mn content ranged from 68 to 166 mg/kg, corresponding to the lowest and highest values of *Brachipodium pinnatum* and *Onobrychis viciaefolia*, respectively. Zn content of browse plants showed a variation from 79 to 103 mg/kg in *Brachipodium pinnatum* and *Onobrychis viciaefolia*, respectively.

### **In vitro digestibility determinations**

The comparison among *in vitro* digestibility coefficients of browse plants using different inoculum source is presented in Table 3 and 4. In general, within each group of plants (legumes, forbs and grasses), digestibility of DM, OM, CP and NDF was similar, and tended to increase with the use of faecal inoculum from yak, in fact only in a few

cases were the minimum digestibility coefficients observed in the samples incubated with rumen fluid from the same animals.

*In vitro* dry and organic matter digestibilities (IVDMD and IVOMD, respectively) showed a higher trend for the forbs group compared to legumes and grasses. Furthermore, the same trend was obtained for *in vitro* crude protein and NDF digestibility (IVCPD and IVNDFD, respectively) coefficients, with the only exception in IVCPD for *Potentilla reptans*.

The overall comparison among digestibility determinations was similar taking into account the use of faecal extract as an alternative microbial inoculum source. In particular, there was no difference for all plant species between rumen and faecal inoculum in IVDMD (0.5511 vs. 0.5522,  $p = 0.143$ ) and IVOMD (0.5751 vs. 0.5785,  $p = 0.102$ ) parameters.

Browse plants seemed in most cases, on average more digestible regarding CP and NDF when incubated with faecal extract than with rumen fluid (Tables 3 and 4). The differences between IVCPD using rumen or faecal inoculum were not statistically significant (0.5668 vs. 0.5679,  $p = 0.202$ ; Table 4) in all collected plants. A similar trend was obtained for IVNDFD estimates which were not significantly different among treatments, even if overall differences tended to be positively increased with the faecal extract.

### **DISCUSSION**

The pastures situated in the Apennines are unique in their species richness, as they are generally comprised of complex plant communities, mainly grasses and, to a less extent, by legumes and other forbs. Their botanical composition is mainly dependent on altitude, climatic and

**Table 3.** Effect of different inoculum source on *in vitro* dry matter and organic matter digestibility coefficients of Appennine browse species

Plant species	IVDMD			IVOMD		
	Rumen fluid	Faecal extract	SED	Rumen fluid	Faecal extract	SED
Legumes						
<i>Lathyrus sativus</i>	0.4912	0.4677	0.0065	0.5182	0.5201	0.0102
<i>Lotus corniculatus</i>	0.6756	0.6813	0.0198	0.6977	0.6991	0.0069
<i>Onobrychis viciaefolia</i>	0.4882	0.4914	0.0172	0.5021	0.5067	0.0077
<i>Trifolium pratense</i>	0.5893	0.5904	0.0113	0.6134	0.6189	0.0165
Forbs						
<i>Achillea millefolium</i>	0.6101	0.6122	0.0059	0.6363	0.6402	0.0118
<i>Potentilla reptans</i>	0.6007	0.6067	0.0105	0.6308	0.6332	0.0105
<i>Teucrium flavum</i>	0.4878	0.4908	0.0067	0.5127	0.5188	0.0067
Grasses						
<i>Brachypodium pinnatum</i>	0.4661	0.4768	0.0126	0.4898	0.4911	0.0126
Overall comparison	0.5511	0.5522	0.0035	0.5751	0.5785	0.0056
			(p = 0.143 )			(p = 0.102)

IVDMD = *In vitro* dry matter digestibility. IVOMD = *In vitro* organic matter digestibility.  
 SED = Standard error of the difference.

edaphic factors.

During vegetative development, a decline in forage quality generally occurs, due to modification of plant morphology, primarily a decrease of the leaf to stem ratio, and to lignification which becomes particularly pronounced during the reproductive and senescent stages (Bovolenta et al., 2008). Moreover, the growth rate of the sward, and modifications of its chemical composition, are strongly affected by seasonal changes of climatic factors.

To study nutritive potential of pastures, a simple method for *in vitro* estimation of feed digestibility has been previously introduced (Mabjeesh et al., 2000), as an

alternative to the traditional method of Tilley and Terry (1963), using the Daisy<sup>II</sup> incubator (Ankom Tech Co., Fairport, NY, USA). This procedure permits a simultaneous incubation of a high number of samples (up to 96 samples per incubation) and does not adversely affect the precision and repeatability of the value obtained (Lattimer et al., 2007).

Estimates achieved using the Daisy<sup>II</sup> incubator used in this trial can be interpreted as estimates of true digestibility of browse plants in the pasture. Since the residue resulting from the incubation *in vitro* for 48 h is a mixture of undigested forage and rumen microorganisms and their

**Table 4.** Effect of different inoculum source on *in vitro* crude protein and neutral detergent fibre digestibility coefficients of Appennine browse species

Plant species	IVCPD			IVNDFD		
	Rumen fluid	Faecal extract	SED	Rumen fluid	Faecal extract	SED
Legumes						
<i>Lathyrus sativus</i>	0.5211	0.5232	0.0032	0.3455	0.3472	0.0102
<i>Lotus corniculatus</i>	0.6807	0.6857	0.0121	0.3503	0.3535	0.087
<i>Onobrychis viciaefolia</i>	0.5002	0.5014	0.098	0.3971	0.3981	0.0123
<i>Trifolium pratense</i>	0.5933	0.5951	0.079	0.4022	0.4027	0.0107
Forbs						
<i>Achillea millefolium</i>	0.6307	0.6311	0.0113	0.3556	0.3568	0.0098
<i>Potentilla reptans</i>	0.6124	0.6135	0.0066	0.3395	0.3376	0.0111
<i>Teucrium flavum</i>	0.5096	0.5088	0.0043	0.3544	0.3545	0.0072
Grasses						
<i>Brachypodium pinnatum</i>	0.4864	0.4841	0.0084	0.3335	0.3346	0.0116
Overall comparison	0.5668	0.5679	0.0051	0.3598	0.3606	0.0062
			(p = 0.202)			(p = 0.155)

IVCPD = *In vitro* crude protein digestibility. IVNDFD = *In vitro* neutral detergent fibre digestibility.  
 SED = Standard error of the difference.

subsequent step by neutral NDF microorganisms and remnants of cell content of forages, so that the cell wall and the time of the degradation of it, determines the value of the *in vitro* digestibility of the substrate. The *in vitro* digestibility with Daisy<sup>II</sup> was not different ( $p>0.05$ ) for the various plants (Tables 3 and 4), possibly due to the order of increasing cell wall content (NDF) and ADF that has been reported and the higher fiber content. Moreover, the similarity between the different repetitions, using faecal extract as inoculum, for all the fodder in the estimation of nutrient digestibility in Daisy<sup>II</sup>, reflects its accuracy, making it comparable to the digestibility values found with the traditional method for many types of forages including grass forage, grass hay, legumes such as alfalfa, silage (Holden, 1999), or concentrates and protein supplements (Mabjeesh et al., 2000). With the use of Daisy<sup>II</sup>, satisfactory accuracy has been reported for this technique for estimating digestibility *in vitro*, even greater than the method of Tilley and Terry (1963). Variation in our work, including all sources of variation, was lower than that found in digestibility estimates for Italian ryegrass and alfalfa (Wilman and Adesogan, 2000; Adesogan, 2005) using the same method. The repeatability obtained, defined as the variation among pitchers, was between 2.2 and 30.6% depending on the type of browse plant.

The repeatability found was higher than reported in other work using the Daisy<sup>II</sup> in the evaluation of different fodder species (Spanghero et al., 2003). Variations in the results of digestibility *in vitro* can be attributed to several factors, such as processing of samples, difference in chemical composition of feed, preparation of buffer solution, handling of equipment and porosity of the filter bags. The Daisy<sup>II</sup>, following the methodology of Goering and Van Soest (1970), is an effective system for estimating *in vitro* digestibility, producing data similar to traditional methodologies also using faecal extract as the microbial inoculum source, enabling a faster processing without negatively affecting the precision of results.

In conclusion, the Daisy<sup>II</sup> incubator system provided a fast, accurate and simple method to determine the digestibility of a large number of browse plant samples compared to conventional methods. Moreover, *in vitro* digestibility measured with the Daisy<sup>II</sup> system had a high repeatability making the procedure fast, easy and economical. Furthermore, the use of faecal extract as the microbial inoculum source for the Daisy<sup>II</sup> incubator may permit this technique to be used in routine laboratory evaluations of the nutritive value of plant species, including those grazed by ruminants, reducing costs and risks associated with the use of fistulated animals and the technical expertise required for rumen fluid collection via the oesophagus. Moreover, *in vivo* studies are needed to optimise grassland use correlated to yak performance and

management.

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