



## The effect of haymaking on the neutral detergent soluble fraction of two intercropped forages cut at different growth stages

Serena Calabrò, Franco Carone, Monica Cutrignelli, Simona D'Urso, Giovanni Piccolo, Raffaella Tudisco, Giovanna Angelino & Federico Infascelli

To cite this article: Serena Calabrò, Franco Carone, Monica Cutrignelli, Simona D'Urso, Giovanni Piccolo, Raffaella Tudisco, Giovanna Angelino & Federico Infascelli (2006) The effect of haymaking on the neutral detergent soluble fraction of two intercropped forages cut at different growth stages, Italian Journal of Animal Science, 5:4, 327-339, DOI: [10.4081/ijas.2006.327](https://doi.org/10.4081/ijas.2006.327)

To link to this article: <https://doi.org/10.4081/ijas.2006.327>



Copyright 2006 Taylor and Francis Group  
LLC



Published online: 01 Mar 2016.



Submit your article to this journal [↗](#)



Article views: 51



Citing articles: 1 View citing articles [↗](#)



# The effect of haymaking on the neutral detergent soluble fraction of two intercropped forages cut at different growth stages

Serena Calabrò<sup>1</sup>, Franco Carone<sup>2</sup>, Monica I. Cutrignelli<sup>1</sup>,  
Simona D'Urso<sup>1</sup>, Giovanni Piccolo<sup>1</sup>, Raffaella Tudisco<sup>1</sup>,  
Giovanna Angelino<sup>2</sup>, Federico Infascelli<sup>1</sup>

<sup>1</sup>Dipartimento di Scienze Zootecniche e Ispezione degli alimenti. Università di Napoli "Federico II", Italy

<sup>2</sup>Dipartimento di Ingegneria agraria e Agronomia del territorio. Università di Napoli "Federico II", Italy

*Corresponding author:* Dr. Serena Calabrò. Dipartimento di Scienze Zootecniche e Ispezione degli alimenti. Università degli Studi di Napoli "Federico II". Via F. Delpino 1, 80137 Napoli, Italy - Tel. +39 081 2536284 - Fax: +39 081 292981 - Email: serena.calabro@unina.it

---

*Paper received March 24, 2006; accepted June 30, 2006*

---

## ABSTRACT

The aim of the paper was to study the fermentation of the neutral detergent soluble fraction (NDS) using a 2x3x2 factorial arrangement of two intercropped forages (barley/broad bean and vetch/oats) collected at three growth stages and preserved by haymaking. Each feed sample and its isolated NDF were fermented *in vitro* and gas production was monitored utilizing the *in vitro* gas production technique (IVGPT). NDS gas yield was determined by the difference between gas from the unfractionated forage and that of its respective NDF. Haymaking decreased gas yield and rate of gas production from the unfractionated forage. Increasing maturity did not change the final gas volume, but reduced the rate of gas production from the NDS fraction. The rate of gas production from the NDS fraction of hay decreased on average by 2.9 ml/h compared with fresh forage. On average, haymaking reduced the gas yield from the NDS fraction by 18.70%. The curve subtraction technique can be used to study the effects of haymaking on the neutral detergent soluble fraction of forages.

*Key Words:* *In vitro* gas production technique (IVGPT), Intercropped forages, Growth stage, Haymaking, Carbohydrate fractions.

## RIASSUNTO

EFFETTO DELLA FIANAGIONE SULLA FRAZIONE SOLUBILE NEL DETERGENTE NEUTRO DI DUE ERBAI RACCOLTI A DIVERSI STADI FENOLOGICI

*Scopo del lavoro è stato lo studio della fermentazione della frazione solubile nel detergente neutro (NDS) utilizzando un modello fattoriale 2 x 3 x 2. Due erbai misti (orzo-favino e veccia-avena) sono stati raccolti a tre diversi stadi fenologici e conservati mediante fienagione. Ciascun campione e la sua relativa frazione NDF sono stati fermentati in vitro e la produzione di gas è stata registrata utilizzando la tecnica in vitro della produzione di gas (IVGPT). Il gas prodotto dall'NDS è stato determinato per differenza tra il foraggio intero e la rispettiva frazione NDF. La fienagione ha provocato una riduzione della produzione di gas e della velocità di fermentazione nel foraggio intero. L'avanzare della maturità della pianta non ha modificato il volume finale di gas, ma ha ridotto la velocità di fermentazione della frazione NDS. La velocità di fermentazione dell'NDS del fieno si è ridotta mediamente di 2,9 ml/h rispetto al foraggio fresco. La fienagione ha ridotto mediamente del 18,70% la produzione di gas nella frazione NDS. Il metodo di*

sottrazione delle curve può essere impiegato per studiare gli effetti della fienagione sulla frazione solubile nel detergente neutro dei foraggi.

Parole chiave: *Tecnica in vitro della produzione di gas (IVGPT), Erbai misti, Stadio vegetativo, Fienagione, Frazioni glucidiche.*

## Introduction

In many Mediterranean countries water stress is the main factor limiting plant growth, since crops are generally managed under rain-fed regimes. In these conditions, small cereal grains (e.g. barley and oats) and vetch are the main crops. They are grown not only for grain and straw production, but also for hay production and grazing. For the latter purposes, they are usually cropped as mixtures (oats and vetch, or barley and vetch) so as to avoid nitrogen fertilization, improve soil physical and chemical properties and obtain higher yields. Mixtures including broad bean are very widely used in the region of Campania (southern Italy). The yield and chemical composition of green forage crops by gramineae and legumes in pure stand and mixed has been extensively studied (Bittante and Andrighetto, 1982; Casoli *et al.*, 1988; Leto *et al.*, 1988, 1989; Pinoso *et al.*, 1995; Piñeiro *et al.*, 2002; Geren *et al.*, 2003; Hoffman and Dér, 2004; Ross, 2004). Moreover, the legume/graminae mixture leads to a more balanced energy/nitrogen ratio in forage for ruminants.

In southern Italy, legumes and gramineae are used for hay production or grazed by small ruminants at various growth stages, providing a considerable part of their protein and energy requirements, especially during the early post partum period. Consequently, information regarding the fermentation kinetics of the organic matter (OM) and its relative fractions in the rumen is extremely useful for formulating balanced diets for high-yielding animals.

The digestion rate of OM and neutral detergent fibre (NDF) may be obtained using standard *in vitro* (Goering and Van Soest, 1970) or *in situ* (Ørskov and McDonald, 1979) techniques. However, such methods cannot be

utilized to evaluate the fermentation of the neutral detergent soluble fraction (NDS) for which the gas curve subtraction method proposed by Schofield and Pell (1995) and used by other authors (Stefanon *et al.*, 1996; Calabrò *et al.*, 2001) seems more suitable.

This paper aims to study the effect of growth stage and haymaking on the fermentation kinetics of the carbohydrate fractions of two intercropped forages, using the *in vitro* gas production technique (IVGPT) described by Theodorou (1993) and the curve subtraction method of Schofield and Pell (1995).

## Material and methods

### *Experimental design*

The effect of forage type, forage maturity and haymaking were assessed using a 2 x 3 x 2 factorial arrangement. Three plots were used for each treatment.

### *Substrates*

Two intercropped forages: barley / broad bean (*Hordeum vulgare* + *Vicia faba minor*, BB) and vetch + oats (*Vicia sativa* + *Avena sativa*, VO) were studied. They were cultivated at a University of Naples research centre situated in S. Angelo dei Lombardi (Avellino, Italy) at 700 m a.s.l. and characterised by cold winters and hot dry summers. The broadcaster was carried out by hand on December 13, 2003 on argillaceous-limestone soil distributing for each specie 50% of the pure seed (barley: 150 kg ha<sup>-1</sup>; broad bean: 220 kg ha<sup>-1</sup>, oats: 140 kg ha<sup>-1</sup>; vetch: 70 kg ha<sup>-1</sup>). The crops received a P and N fertilization of 150 kg P<sub>2</sub>O<sub>5</sub> ha<sup>-1</sup> and 60 kg N ha<sup>-1</sup>, respectively (50% before the seeds and the remaining 50% when the gramineae were in the growing phase). Starting from May 2004, forages were harvested at three different growth stages, for grass and legumes, respectively:

Table 1. Chemical composition (% DM) of forages.

Forage	Stage	DM	Crude protein	NDF	NDSC	ADL <sup>1</sup>	ADF	ADL
				Fresh				
	1°	16.1	17.7 <sup>A</sup>	48.2	20.5 <sup>C</sup>	8.38	26.8 <sup>b</sup>	4.04
	2°	22.1	14.5 <sup>Ba</sup>	48.9	24.8 <sup>B</sup>	9.37	28.3 <sup>a</sup>	4.58
	3°	22.8	14.0 <sup>Bb</sup>	47.8	26.9 <sup>A</sup>	10.2	26.5 <sup>b</sup>	4.86
Barley-broad bean				Hay				
	1°	92.21	14.3 <sup>A</sup>	53.1 <sup>B</sup>	20.0 <sup>B</sup>	8.94 <sup>b</sup>	34.4	4.65 <sup>B</sup>
	2°	91.45	12.1 <sup>Ba</sup>	53.8 <sup>Bb</sup>	22.1 <sup>A</sup>	8.83 <sup>b</sup>	35.2	4.75 <sup>ABb</sup>
	3°	91.88	11.6 <sup>Bb</sup>	55.5 <sup>Aa</sup>	19.4 <sup>B</sup>	11.4 <sup>a</sup>	34.3	6.33 <sup>Aa</sup>
				Fresh				
	1°	21.4	13.8 <sup>A</sup>	47.9 <sup>B</sup>	25.6 <sup>a</sup>	7.79	29.5	4.21 <sup>b</sup>
	2°	22.9	11.3 <sup>Ba</sup>	51.1 <sup>A</sup>	25.8 <sup>a</sup>	10.5	32.8	5.38 <sup>ab</sup>
	3°	25.6	10.8 <sup>Bb</sup>	52.0 <sup>A</sup>	27.1 <sup>b</sup>	10.6	35.4	5.52 <sup>a</sup>
Vetch-oats				Hay				
	1°	92.09	11.4 <sup>A</sup>	54.6 <sup>C</sup>	20.8 <sup>A</sup>	9.98	38.3	5.45 <sup>B</sup>
	2°	92.30	8.21 <sup>B</sup>	62.5 <sup>A</sup>	17.9 <sup>B</sup>	9.14	41.9	5.71 <sup>A</sup>
	3°	93.22	9.25 <sup>C</sup>	59.0 <sup>B</sup>	21.2 <sup>A</sup>	10.1	42.6	5.98 <sup>A</sup>
MSE		-	3.04	8.84	2.43	3.67	7.01	0.510
Effect of Forage		-	0.001	0.001	0.150	0.3890	0.01	0.0430
Effect of Storage			0.001	0.01	0.001	0.8045	0.01	0.0073
Effect of Maturity		-	0.001	0.05	0.01	0.0491	0.05	0.0061

NDSC: neutral detergent soluble carbohydrates = carbohydrates of NDS (estimated fraction).

<sup>1</sup>ADL: estimated content of NDF fraction (ADL / NDF × 100).

Means with different small or capital letters in the column differ significantly at  $P < 0.05$  or  $< 0.01$ , respectively.

end of booting and vegetative growth (1<sup>st</sup> stage), heading and beginning of flowering (2<sup>nd</sup> stage), and the beginning of the milky maturation of the grain and pod formation (3<sup>rd</sup> stage).

#### Hay preparation

Each fresh forage was picked at random from three plots. From each plot a sample was immediately collected and the remainder was left in the field until the wilting was completed. 36 samples were thus obtained.

#### Forages analysis

All substrates (fresh forages and hay) were

air dried at 65°C and milled to pass a 1 mm screen (Brabender Wiley mill, Brabender OHG Duisburg, Germany). Dry matter (DM), ash, crude protein (CP) and crude fibre (CF) contents were determined according to AOAC (2000) procedures (ID members: 930.04, 930.05, 977.02 and 930.10, respectively). Neutral detergent fibre (NDF) was determined by boiling for 1 hour 0.5 g sample in 100 ml of neutral detergent plus 50 µl of heat stable  $\alpha$ -amylase (ANKOM Technology) and 0.5 g of sodium sulphite (Van Soest *et al.*, 1991). Acid detergent fibre (ADF) and lignin (ADL) were determined according to Goering and Van Soest (1970).

Table 2. Least squares means representing the main treatment effects for the unfractionated forage samples.

Class	dOM (%)	OMCV (ml/g)	Yield (ml/g)	A (ml/g)	B (h)	C	t <sub>max</sub> (h)	R <sub>max</sub> (ml/h)	pH
Effect of forage species									
Barley-broad bean	81.8 <sup>A</sup>	309	380 <sup>b</sup>	299 <sup>B</sup>	17.1 <sup>B</sup>	1.52 <sup>A</sup>	6.03	11.1 <sup>A</sup>	6.49
Vetch-oats	78.0 <sup>B</sup>	311	396 <sup>a</sup>	314 <sup>A</sup>	21.6 <sup>A</sup>	1.40 <sup>B</sup>	5.87	9.36 <sup>B</sup>	6.49
Effect of growth stage									
1° stage	82.6 <sup>A</sup>	319 <sup>A</sup>	391 <sup>a</sup>	308 <sup>a</sup>	17.8 <sup>B</sup>	1.50	5.98	11.0 <sup>A</sup>	6.51
2° stage	80.1 <sup>B</sup>	319 <sup>A</sup>	400 <sup>Aa</sup>	314 <sup>Aa</sup>	19.5 <sup>Ab</sup>	1.45	5.99	10.4 <sup>AB</sup>	6.47
3° stage	77.0 <sup>C</sup>	291 <sup>B</sup>	374 <sup>Bb</sup>	297 <sup>Bb</sup>	20.7 <sup>Aa</sup>	1.42	5.89	9.32 <sup>B</sup>	6.48
Effect of preservation method									
Fresh	80.1	315 <sup>a</sup>	392	312 <sup>b</sup>	17.0 <sup>B</sup>	1.39 <sup>B</sup>	4.45 <sup>B</sup>	11.6 <sup>A</sup>	6.54 <sup>A</sup>
Hay	79.7	304 <sup>b</sup>	385	301 <sup>a</sup>	21.6 <sup>A</sup>	1.53 <sup>A</sup>	7.45 <sup>A</sup>	8.82 <sup>B</sup>	6.43 <sup>B</sup>
MSE	5.63	332	668	345	2.77	0.020	2.53	1.79	0.0172

dOM: OM degradability; OMCV: gas production related to incubated OM; Yield: gas production related to degraded OM; A: potential gas production; B: time at which A/2 was formed; C: constant determining the curve sharpness; t<sub>max</sub>: time at which maximum rate was reached. R<sub>max</sub>: maximum rate.

MSE = mean square error. Means with different small or capital letters in the fraction differ significantly at  $P < 0.05$  or  $P < 0.01$ , respectively.

#### Neutral detergent fibre preparation

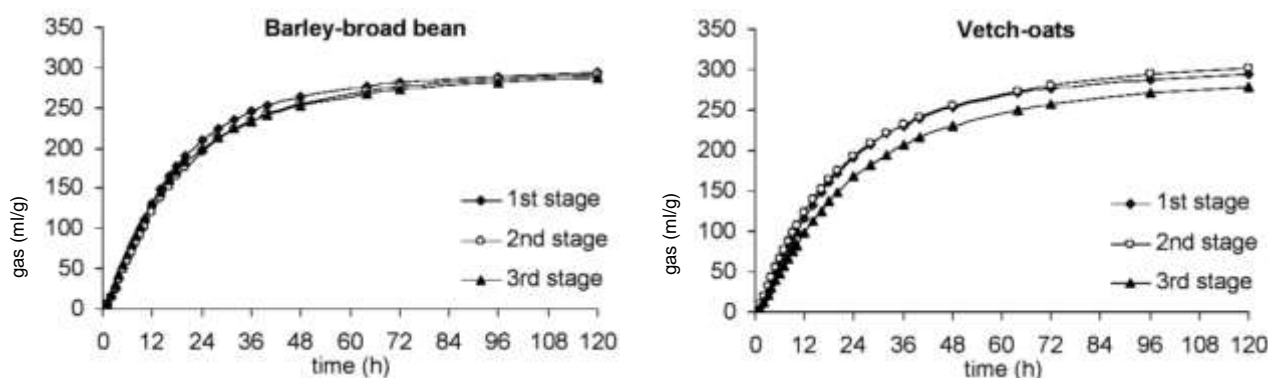
The NDF fraction for fermentation studies was prepared according to Van Soest *et al.* (1991) using  $\alpha$ -amylase and sodium sulphite as described previously. The pooled extracted fibre was washed several times with hot distilled water, filtered through a 37  $\mu$ m mesh nylon screen and rinsed with 100 ml of ethanol and acetone. According to Schofield and Pell (1995) the fibre was vacuum dried and incubated at 39°C with 1 M ammonium sulphate to remove any trace of ionically bound detergent; then it was filtered, washed in a vacuum and dried at 50°C.

#### In vitro gas production

Whole forage (WF) and its isolated NDF were incubated (1.049  $\pm$  0.0250 g) at 39°C under anaerobic conditions with 75 ml of bicarbonate-phosphate buffer, 4 ml of reducing agent and 10 ml of rumen fluid, obtai-

ned from two adult crossbreed (Delle Langhe x Comisana) sheep (approximate 50 kg LW). They were fed 1.4 kg DM daily, supplied in two equal meals of a diet consisting in 60% grass hay and 40% concentrate (beet pulp, wheat middling, wheat flour middling, corn gluten feed and soya bean meal). The rumen fluid was collected before the morning feeding and filtered through various layers of gauze. Fermentation was carried out using 120 ml serum bottles sealed with butyl rubber stoppers and aluminium crimps. Four bottles containing no substrate were incubated as blanks to correct for OM disappearance, and gas and volatile fatty acids (VFA) production. Gas production was recorded 21 times, at 2-24 intervals during the period of fermentation (120 h); a pressure transducer and a LED Digital read-out voltmeter (Cole and Parmer Instrument Co, Illinois, USA) were used to measure the

Figure 1. *In vitro* cumulative gas production over time of the unfractionated fresh forage at the three stages of maturity for barley-broad bean and vetch-oats.



head space gas pressure of fermenting cultures. The voltmeter was calibrated by the manufacturer to read units of pressure (PSI). The transducer was modified to be connected to a disposable plastic syringe of 5, 10, 20 or 60 ml capacity according to the volume of gas to be measured. A detailed description of the equipment used for the gas production measurements is given by Theodorou *et al.* (1994). Each forage and its NDF fraction were fermented in two separate trials 1 wk apart.

Fermentation was stopped at 120 h, after which the pH of the fermenting liquor was measured (Alessandrini Instrument glass electrode; mod. JENWAY 3030) and a sample (5 ml) was collected for volatile fatty acid analysis. Then the contents of each bottle were filtered through pre-weighed fibre crucibles (Schott-Duran, Mainz, Germany, porosity #2) under vacuum. Incubation residues were first oven dried at 103°C for 24 h and then ashed at 550°C to determine organic matter (dOM) or NDF (dNDF) disappearance. The sample for VFA analysis was centrifuged twice at 12000 g for 10 minutes at 4°C; after centrifugation 1 ml of supernatant was taken, to which 1 ml of oxalic acid 0.06 M was added. The VFA were measured by gas chromatography (Thermo Quest mod. 8000<sup>top</sup>, FUSED SILICA capillary column 30 m x 0.25 mm x 0.25 mm film thickness) including acetate, propionate, butyrate, iso-butyrate, valerate and iso-valerate as external standards.

#### Curve subtraction

To estimate the fermentation rate of the NDS fraction the curve subtraction approach was utilized (Schofield and Pell, 1995); it requires the fermentation of an unfractionated forage and its respective NDF fraction. The WF average gas production and that of its respective NDF were adjusted to represent the amount of each fraction present in 1 g of WF organic matter. Then the gas volumes produced by the NDS fraction at each time were calculated as the difference between the gas yields of the unfractionated sample and its respective NDF.

#### Calculation of kinetic parameters

For curve fitting, the gas profile (as ml of gas produced per g OM in time) were fitted to the monophasic version of the model described by Groot *et al.* (1996) as follows:

$$G \text{ (ml/g)} = A/(1+B/\text{time})^C$$

where  $G$  (ml/g) = total gas produced (for WF related to the incubated OM, while for NDF and NDS, respectively, it represents the gas related to the amount of NDF and NDS present in 1 g of OM whole forage),  $A$  (ml/g) = asymptotic gas production,  $B$  (h) = time at which  $A/2$  was reached, and  $C$  = switching characteristic of the curve. The maximum rate of gas production ( $R_{\max}$ ) and the time at which it occurs ( $T_{\max}$ ), were calculated according to following equations (Bauer *et al.*, 2001):

Table 3. Least squares means representing the main treatment effects for the NDF fraction.

Class	dNDF %	gas (ml/g)	gas* (ml)	Yield (ml/g)	A (ml/g)	B (h)	C	t <sub>max</sub> (h)	R <sub>max</sub> (ml/h)	pH
Effect of forage species										
Barley-broad bean	69.6	274 <sup>B</sup>	134 <sup>B</sup>	421 <sup>B</sup>	139 <sup>B</sup>	32.9 <sup>B</sup>	2.42 <sup>A</sup>	22.7	3.07 <sup>b</sup>	6.31
Vetch-oats	68.2	288 <sup>A</sup>	151 <sup>A</sup>	449 <sup>A</sup>	160 <sup>A</sup>	34.6 <sup>A</sup>	2.22 <sup>B</sup>	22.0	3.22 <sup>a</sup>	6.28
Effect of growth stage										
1° stage	73.8 <sup>A</sup>	301 <sup>Aa</sup>	147 <sup>A</sup>	429 <sup>B</sup>	152 <sup>A</sup>	31.6 <sup>Bb</sup>	2.39 <sup>A</sup>	21.7	3.46 <sup>A</sup>	6.25 <sup>Bb</sup>
2° stage	68.2 <sup>B</sup>	287 <sup>Ab</sup>	150 <sup>A</sup>	463 <sup>A</sup>	156 <sup>A</sup>	33.0 <sup>Ba</sup>	2.37 <sup>A</sup>	22.4	3.38 <sup>A</sup>	6.28 <sup>Ba</sup>
3° stage	64.8 <sup>C</sup>	256 <sup>B</sup>	131 <sup>B</sup>	413 <sup>B</sup>	140 <sup>B</sup>	36.6 <sup>A</sup>	2.20 <sup>B</sup>	23.0	2.60 <sup>B</sup>	6.35 <sup>A</sup>
Effect of preservation method										
Fresh	68.6	281	134 <sup>B</sup>	437	141 <sup>B</sup>	32.3 <sup>B</sup>	2.22 <sup>B</sup>	20.6 <sup>B</sup>	3.02 <sup>B</sup>	6.34 <sup>A</sup>
Hay	69.2	281	151 <sup>A</sup>	433	158 <sup>A</sup>	35.2 <sup>A</sup>	2.43 <sup>A</sup>	24.1 <sup>A</sup>	3.28 <sup>A</sup>	6.25 <sup>B</sup>
MSE	12.74	445	79.9	1171	98	3.76	0.0385	5.44	0.076	0.0094

dNDF: NDF degradability; \*gas production related to the amount of NDF in 1 g of OM; A: potential gas production; B: time at which A/2 was formed; C: constant determining the curve sharpness; t<sub>max</sub>: time at which maximum rate was reached. R<sub>max</sub>: maximum rate.

MSE = mean square error. Means with different small or capital letters in the fraction differ significantly at  $P < 0.05$  or  $P < 0.01$ , respectively.

## Results

### Chemical composition

The chemical composition of the forages is shown in Table 1. The main effects (forage, preservation method and maturity) significantly influenced most of the parameters. In particular, for both fresh forages, lignin increased and crude protein decreased with the growing stage. For the protein the differences were always statistically significant ( $P < 0.01$ ). Regarding the structural carbohydrates, for vetch-oats, ADF increased with advancing age (29.5 vs 32.8 vs 35.4 % DM, 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> stage, respectively), whereas barley-broad bean showed a higher ADF content in the 2<sup>nd</sup> than in the 3<sup>rd</sup> stage, due to the presence of the grain, as reported for barley forage by Hadjipanayiotou *et al.* (1996) and INRA (1988). For vetch-oats fresh forages, NDF

$$R_{\max} \cdot (\text{ml/h}) = (A \cdot C^B) \cdot B \cdot [T_{\max}^{-(B-1)}] / [(1 + C^B) \cdot (T_{\max}^{-B})^2]$$

$$T_{\max} \text{ (h)} = C \cdot [(B-1)/(B+1)]^{(1/B)}$$

where A, B and C denote the same as above.

### Statistical analysis

The chemical composition parameters, the fermentation characteristics (gas and VFA produced, OM degradability and pH) and the fitted parameters for WF, NDF and NDS fractions were subjected to analysis of variance to detect the forage, preservation method and maturity effects. In the model all the interactions were included. Moreover, for the fermentation characteristics, in order to allow for rumen liquor variability the fermentation day was included in the model. For all the statistical analyses the GLM procedure of SAS (2000) was utilised.

Figure 2. Fermentation rate over time of the unfractionated fresh forage at the three stages of maturity for barley-broad bean and vetch-oats.

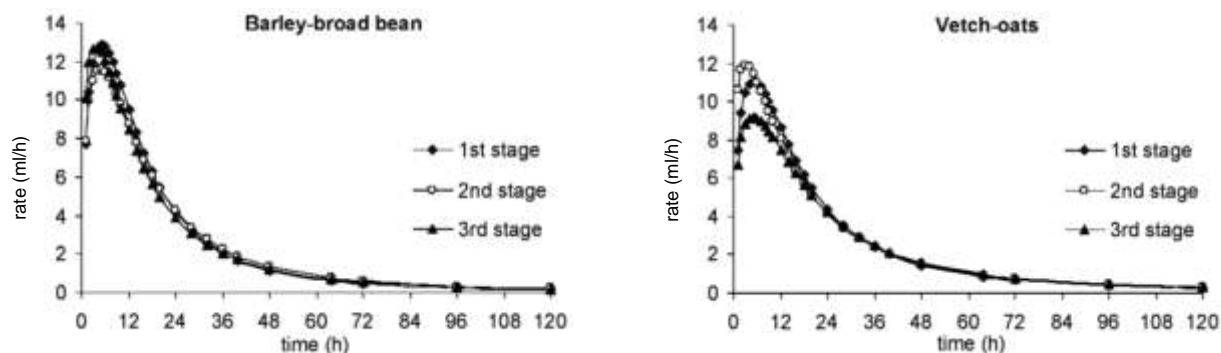
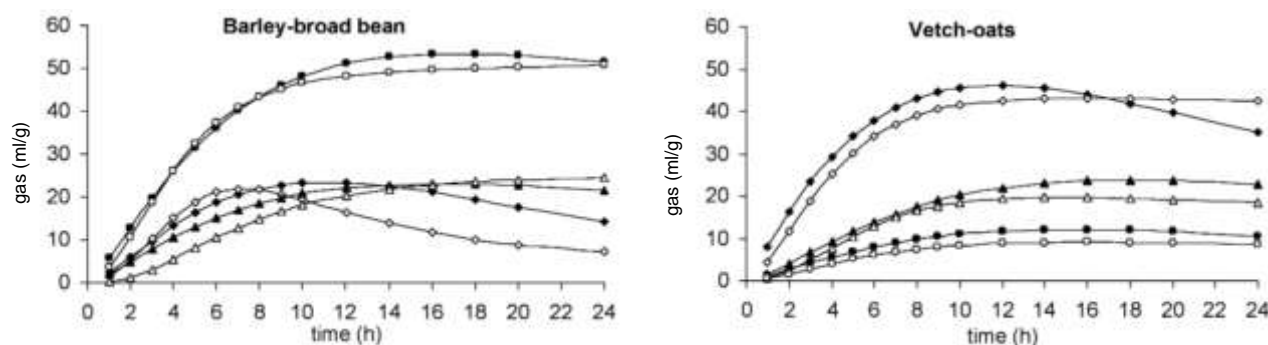


Figure 3. The discrepancy in gas produced by fermentation of the fresh unfractionated forage and hay material for barley-broad bean and vetch-oats.



Calculations from the unfractionated forages (filled symbols) and the neutral detergent soluble (NDS) (open symbols) presented for tree maturity stage: 1° (triangle), 2° (square) and 3° (circle). Gas production for the NDS was calculated by difference between the gas yield of unfractionated forage and the gas yield of its respective NDF preparation. Gas volumes are based on the millilitres of gas from 1 g of unfractionated forage OM.

content was affected by growth stage.

Comparing the mean of the two inter-cropped forages, barley-broad bean showed higher crude protein content (14.0 vs 10.8 % DM) and lower parietal carbohydrates (NDF: 51.2 vs 54.5 % DM) and lignin content (4.87 vs 5.21 % DM) than vetch-oats ( $P < 0.01$ ).

Proceeding from fresh forage to hay, as expected, we noted some variation due to the preservation techniques: a decrease in protein (13.7 vs 11.1 % DM) and an increase in structural carbohydrate (NDF: 49.3 vs 56.4 % DM) and lignin (4.77 vs 5.48 % DM) contents ( $P < 0.01$ ) (CP, NDF and lignin data are mean values calculated by those reported in table 1).

#### Fermentation characteristics

Tables 2, 3 and 4 show the fermentation characteristics of the unfractionated forage sample and the NDF and NDS fractions, respectively. Barley-broad bean showed higher OM and NDF degradability compared to vetch-oats; moreover, these values decrease significantly in advancing phenological stage ( $P < 0.01$ ).

When the gas production of the NDF fraction was adjusted to represent the amount of NDF fraction present in 1 g of WF organic matter (Table 3) the following results were obtained: VO > BB (151 vs 134 ml;  $P < 0.01$ ); hay > fresh (151 vs 134 ml;  $P < 0.01$ ); 1<sup>st</sup> and 2<sup>nd</sup> stages > 3<sup>rd</sup> stage (150 and 147 vs 131 ml;



Table 4. Least squares means representing the main treatment effects for the NDS fraction.

	gas (ml/g)	A (ml/g)	B (h)	C	t <sub>max</sub> (h)	R <sub>max</sub> (ml/h)
Effect of forage species						
Barley-broad bean	148 <sup>a</sup>	155 <sup>a</sup>	8.67	2.10 <sup>a</sup>	5.03 <sup>a</sup>	12.09 <sup>a</sup>
Vetch-oats	133 <sup>b</sup>	138 <sup>b</sup>	8.90	1.79 <sup>b</sup>	4.13 <sup>b</sup>	10.22 <sup>b</sup>
Effect of growth stage						
1 <sup>o</sup> stage	145	149	8.13	2.17 <sup>a</sup>	4.76	12.6 <sup>A</sup>
2 <sup>o</sup> stage	138	144	8.49	1.81 <sup>b</sup>	4.25	11.1 <sup>AB</sup>
3 <sup>o</sup> stage	139	146	9.75	1.85 <sup>b</sup>	4.74	9.83 <sup>B</sup>
Effect of preservation method						
Fresh	155 <sup>A</sup>	162 <sup>A</sup>	8.20	1.83	3.88 <sup>B</sup>	12.6 <sup>A</sup>
Hay	126 <sup>B</sup>	131 <sup>B</sup>	9.37	2.06	5.28 <sup>A</sup>	9.70 <sup>B</sup>
MSE	765	1075	10.05	0.279	3.03	13.01

Gas: production related to the amount of NDS in 1 g of OM A: potential gas production; B: time at which A/2 was formed; C: constant determining the curve sharpness; t<sub>max</sub>: time at which maximum rate was reached. R<sub>max</sub>: maximum rate.

MSE = mean square error. Means with different small or capital letters in the fraction differ significantly at P < 0.05 or P < 0.01, respectively.

P < 0.01). For the first two results the NDF contents undoubtedly contributed, while the differences between the stages were caused by the lignin content of the NDF (8.80, 9.46 and 10.58 % DM, for 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> stage, respectively) that also significantly influenced (P < 0.01) OM degradability.

The final pH after 120 h of incubation (Tables 2 and 3) shows that the buffering capacity of the medium was sufficient to maintain the pH within the 6.2 to 6.8 range necessary to ensure a linear relationship between VFA and gas production (Beuvink and Spoelstra, 1992).

The volatile fatty acids, produced after 120 h of fermentation by the whole forage and the NDF fractions, are reported in tables 5 and 6. For both, the values are not statistically different in the two intercropped forages. However, some significant diffe-

rences were found due to maturity and storage. In any case, there is a good correlation between gas and total volatile fatty acids produced, both for WF (r<sup>2</sup> = 0.70; P < 0.01) and NDF (r<sup>2</sup> = 0.93; P < 0.01).

#### *Kinetics analysis of gas production*

The kinetics parameters (B, t<sub>max</sub> and R<sub>max</sub>) were in part affected by the main effect (forage, maturity, preservation) for WF, NDF and NDS (Tables 2, 3 and 4, respectively). In particular, the trend in maximum fractional rate (R<sub>max</sub>, ml/h) for whole forage and NDS was: BB > VO (P < 0.01 for WF and P < 0.05 for NDS), 1<sup>st</sup> stage > 3<sup>rd</sup> stage (P < 0.01), Fresh > Hay (P < 0.01).

Figure 1 shows the *in vitro* gas production profiles of the two intercropped unfractionated fresh forages at three stages of maturity.

The gas production data were described

Table 5. Least squares means representing the main treatment effects for the unfractionated forage for pH and volatile fatty acids.

	Acetate	Propionate	Butyrate	Tot VFA	A/P	(A+B)/P
	(Mm/g)					
Effect of forage species						
Barley-broad bean	55.54	25.37	9.63	90.54	2.24	2.62
Vetch-oats	55.85	25.64	8.88	90.36	2.19	2.54
Effect of growth stage						
1° stage	56.46	27.57 <sup>A</sup>	9.96 <sup>A</sup>	93.99 <sup>A</sup>	2.06 <sup>B</sup>	2.43 <sup>B</sup>
2° stage	57.10	26.30 <sup>AB</sup>	7.99 <sup>C</sup>	91.40 <sup>ABa</sup>	2.18 <sup>ABb</sup>	2.49 <sup>BC</sup>
3° stage	53.52	22.64 <sup>C</sup>	9.80 <sup>AB</sup>	85.97 <sup>Bb</sup>	2.39 <sup>Aa</sup>	2.82 <sup>A</sup>
Effect of preservation method						
Fresh	57.72 <sup>A</sup>	27.09 <sup>A</sup>	9.81 <sup>a</sup>	94.62 <sup>A</sup>	2.15	2.52
Hay	53.67 <sup>B</sup>	23.92 <sup>B</sup>	8.70 <sup>b</sup>	86.29 <sup>B</sup>	2.28	2.65
MSE	15.03	15.03	3.503	69.7	0.084	0.0691

VFA: volatile fatty acids; A/P: acetate propionate ratio; (A+B)/P: (acetate + butyrate)/propionate.

very well by the model ( $R^2$  ranged from 0.9954 to 0.9996).

The three stages of barley-broad bean showed a superimposable trend of the gas production and fermentation rate. By contrast, for vetch-oats the 3<sup>rd</sup> stage showed at all times lower gas production; the same trend was observed for the fermentation rate compared to the other two stages ( $R_{max}$ : 12.0 and 11.1 > 9.09 ml/h, for 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> stage, respectively;  $P < 0.01$ ).

## Discussion

### Forage effects

The two legume-graminae mixtures showed some differences in fermentation kinetics, undoubtedly due to differences in the chemical composition (e.g. higher crude protein and lower parietal carbohydrates and lignin in BB than in VO).

The unfractionated forage of barley-broad bean was more digestible than vetch-oats ( $P < 0.01$ ), but final gas volumes were the same. However, VFA production and the

acetate:propionate ratio were similar, according to the double fate of degraded OM: gas and volatile fatty acid production and microbial biomass production (Williams, 2000).

### Maturity effects

For both intercropped forages, fresh and hay, plant maturity changed some characteristics of the chemical composition (i.e. DM and lignin increased and crude protein decreased) and hence some fermentation characteristics. In particular, advancing maturity considerably reduced OM and NDF digestibility, gas and VFA production. The effect of plant maturity on *in vitro* and *in situ* degradability has been studied by other authors: in line with our findings, Crovetto *et al.* (1997) found a significant ( $P < 0.05$ ) decrease in DM and NDF *in situ* effective degradability for two cultivars of Italian ryegrass harvested at six stages of maturity; Doane *et al.* (1997) reported a decrease in gas and VFA production and considerable NDF *in vitro* degradability with increasing maturity for alfalfa, bromegrass and orchardgrass; Stefanon *et al.* (1997) studied *in vitro* the

Table 6. Least squares means representing the main treatment effects for the NDF fraction for pH and volatile fatty acids.

Class	Acetate	Propionate (Mm/g)	Butyrate	Tot VFA	A/P	(A+B)/P
Effect of forage species						
Barley-broad bean	54.52	22.77	7.05	84.34	2.53	2.84
Vetch-oats	55.82	25.02	6.47	87.31	2.28	2.53
Effect of growth stage						
1° stage	58.34 <sup>A</sup>	26.99 <sup>Aa</sup>	7.87 <sup>Aa</sup>	93.19 <sup>Aa</sup>	2.22	2.51
2° stage	56.20 <sup>ABa</sup>	24.17 <sup>ABb</sup>	6.38 <sup>ABb</sup>	86.75 <sup>ABb</sup>	2.39	2.65
3° stage	50.97 <sup>Bb</sup>	20.51 <sup>Bc</sup>	6.03 <sup>B</sup>	77.51 <sup>C</sup>	2.61	2.90
Effect of preservation method						
Fresh	56.12	23.11	6.91	86.13	2.48	2.77
Hay	54.22	24.62	6.61	85.52	2.33	2.60
MSE	75.1	23.52	5.31	94.08	0.596	0.533

VFA: volatile fatty acids; A/P: acetate propionate ratio; (A+B)/P: (acetate + butyrate)/propionate .

maturity effect on DM and NDF degradability and reported that the less mature forages were more digestible for both alfalfa and brome.

For fresh vetch-oats, maturity affected the fermentation kinetics. In particular, the 3<sup>rd</sup> stage showed less gas production and a lower maximum fermentation rate over time compared to the other two stages. These results are hard to explain: despite the highest lignin content the 3<sup>rd</sup> stage of VO also presents the lowest neutral detergent soluble carbohydrates. Stefanon *et al.* (1997) using the IVGPT reported that changes in kinetic parameters with increasing maturity for alfalfa and brome were somewhat ambiguous.

#### *Haymaking effects*

As expected, proceeding from fresh forage to hay some variation due to the preservation techniques were observed: a decrease in protein and an increase in structural carbohydrate and lignin contents.

In the unfractionated forage, haymaking had no influence on OM digestibility, but gas and VFA production were significantly

( $P < 0.05$  and  $P < 0.01$ , respectively) lower in hay than in fresh forage. Because VFA production is associated with the release of  $\text{CO}_2$  from both the microbial metabolism and the reaction of acid with the bicarbonate buffer, the differences in VFA production account for the differences in gas production.

When compared with fresh forage, hay-making reduced the maximum fractional rate ( $R_{\max}$ ) also reported in a previous paper (Calabrò *et al.*, 2005). The faster kinetics of fresh forages described is confirmed by the tendency of parameter B (the time at which A/2 is reached) which is lower in the fresh forage than hay.

Differences in gas production due to hay-making may be presented in two ways: 1) unfractionated fresh forage – unfractionated hay; 2) fresh NDS – hay NDS. Assuming that the NDF fraction shows the same behaviour whether isolated or within the unfractionated forage (Schofield and Pell, 1995; Calabrò *et al.*, 2001), the following equations, related to the gas production, can be written:

$$WF_{\text{fresh}} - WF_{\text{hay}} = (NDF + NDS)_{\text{fresh}} - (NDF + NDS)_{\text{hay}} \quad (1)$$

$$WF_{\text{fresh}} - WF_{\text{hay}} = NDF_{\text{fresh}} + NDS_{\text{fresh}} - NDF_{\text{hay}} - NDS_{\text{hay}} \quad (2)$$

$$WF_{\text{fresh}} - WF_{\text{hay}} = NDS_{\text{fresh}} - NDS_{\text{hay}} + (NDF_{\text{fresh}} - NDF_{\text{hay}}) \quad (3)$$

Consequently, the subtraction curve between the gas production of the fresh whole forage and the hay whole forage differs from the subtraction curve between the gas production of the fresh forage NDS and hay NDS by a constant amount equal to  $(NDF_{\text{fresh}} - NDF_{\text{hay}})$ .

The curves in Figure 3 represent the discrepancy in gas produced by fermentation of forage and hay (fresh - hay) calculated either from unfractionated forages or from the neutral detergent soluble fraction (NDS) for the three stages of the two intercropped forages. These curves are nearly parallel according to the relation reported in the equations (1), (2) and (3). Similar results were obtained by Doane *et al.* (1997) who studied the effect of ensiling on the neutral detergent soluble fraction of forages at different maturity stage. This indicates that the fermentation of the fibre within the unfractionated forage and the isolated NDF were similar, as also reported elsewhere (Schofield and Pell, 1995; Stefanon *et al.*, 1996; Doane *et al.*, 1997; Calabrò *et al.*, 2001).

The differences in gas production between fresh and hay forages were greater in the 3<sup>rd</sup> stage for barley-broad bean and in the 2<sup>nd</sup> stage for vetch-oats, and when there were greater differences between the NDSC contents. Moreover, for the 3<sup>rd</sup> stage of BB, haymaking also determined a higher reduction of the gas production rate (ml/h) compared to the 1<sup>st</sup> and 2<sup>nd</sup> stages (4.9 vs 1.5 and 2.1 ml/h, respectively). A similar result was observed for the 2<sup>nd</sup> stage of VO, where storage caused a higher reduction of the fermentation rate compared to the 1<sup>st</sup> and 3<sup>rd</sup> stages (3.75 vs 2.74 and 1.69 ml/h, respectively).

Little is known about the digestion kinetics of the neutral detergent-soluble fraction, because most *in vitro* kinetic methods

have studied the disappearance of insoluble cell wall components and, in addition, gravimetric techniques do not usually measure the soluble fraction. However, knowledge of the degradation characteristics of both fractions is very important to formulate balanced diets for high-yielding animals. For this purpose the curve subtraction method is useful, as reported by many authors (Schofield and Pell, 1995; Stefanon *et al.*, 1996; Calabrò *et al.*, 2001). In this study, curve subtraction proved a reliable method to also describe changes in the soluble fraction of forages due to storage, confirming the findings of Doane *et al.* (1997) and Calabrò *et al.* (2005). The reduction in gas production from the fermentation of the unfractionated forages due to haymaking was similar to the reduced gas yield from the fermentation of the NDS fractions. The correlation between the two values was high (r ranged from 0.75 to 1.00). This suggests that the main reason for reduced gas production due to haymaking was the decrease in NDS content. Besides, due to haymaking, the contribution of the NDF fraction to gas production increases while that of the NDS decreases.

The NDS fraction fermented rapidly and, in contrast with the results of Stefanon *et al.*, (1996) and Doane *et al.* (1997), maturity had no effect on the gas volume produced, but the maximum fermentation rate decreased (Table 4). Our results show that haymaking reduces the NDS maximum fractional rate of fermentation by 2.9 ml/h. What undoubtedly contributed to this result was the loss of nutrients (in particular soluble carbohydrates and the nitrogen component) during haymaking arising from the action of plant and microbial enzymes and mechanical damage (McDonald *et al.*, 1992).

## Conclusions

Comparing the two legume-graminae mixtures, barley-horse bean had the better nutritive value (i.e. higher protein and lower struc-

tural carbohydrates) and better fermentation characteristics (i.e. higher degradability and faster kinetics). On advancing maturity, although some differences were found in chemical composition, the three forages of barley-broad bean cut at different stages showed a similar kinetics. By contrast, for vetch-oats the third stage showed a slower kinetics. As expected, haymaking modified some chemical characteristics, and led to a decrease in gas and VFA production and a slower fermentation kinetics.

The curve subtraction approach allows the fermentation kinetics of the soluble carbohydrate fraction to be measured. Consequently, it was possible to ascertain the effects of the variation in chemical composition due to the vegetative stage and to haymaking. Moreover, this method can contribute to choose the best vegetative stage at which to cut the plant to obtain hay successfully. In our conditions, in order to limit storage losses, it would be advisable not to use the forage cut at the beginning of the milky maturation of the grain and pod formation (3<sup>rd</sup> stage) for barley-broad bean and the heading and beginning of flowering (2<sup>nd</sup> stage) for vetch-oats.

*The authors would like to thank Mrs. Maria Ferrara for her technical collaboration.*

*Some of the results displayed in this paper were discussed at the 16<sup>th</sup> ASPA Congress (Torino - Italy, June 28-30, 2005).*

## REFERENCES

- AOAC, 2000. Official Methods of Analysis. 15<sup>th</sup> ed. Association of Official Analytical Chemists, Arlington, VA, USA.
- BAUER, E., WILLIAMS B.A., VOIGT, C., MOSENTHIN, R., VERSTEGEN, M.W.A., 2001. Microbial activities of faeces from unweaned and adult pigs, in relation to selected fermentable carbohydrates. *Anim. Sci.* 73:313-322.
- BEUVINK, J.M.W., SPOELSTRA, S.F., 1992. Interactions between substrate, fermentation, end-products, buffering system and gas production upon fermentation of different carbohydrates by mixed rumen microorganisms *in vitro*. *Appl. Microbiol. Biot.* 37:505.
- BITTANTE, G., ANDRIGHETTO, I., 1982. Conservazione del *Lolium Multiflorum* Lam. Modificazioni quali-quantitative nella fienagione e nell'insilamento. *Zoot. Nutr. Anim.* 8:41-51.
- CALABRÒ, S., INFASCELLI, F., BOVERA, F., MONIELLO, G., PICCOLO, V., 2001. *In vitro* degradability of three forages: fermentation kinetics and gas production of NDF and neutral detergent soluble-fraction of forages. *J. Sci. Food Agr.* 82:222-229.
- CALABRÒ, S., CUTRIGNELLI, MI, BOVERA, F, PICCOLO, G, INFASCELLI, F., 2005. *In vitro* fermentation kinetics of carbohydrate fractions of fresh forage, silage and hay of *Avena sativa*. *J. Sci. Food Agr.* 85:1838-1844.
- CASOLI, C., DURANTI, E., FALCINELLI, M., 1988. Utilizzazione digestiva di *Dactylis glomerata* L. allo stato verde e dopo conservazione mediante insilamento e fienagione. *Zoot. Nutr. Anim.* 14:261-266.
- CROVETTO, G.M., SANDRUCCI, A., TAMBURINI, A., BORREANI, G., 1997. Degradabilità ruminale di loglio italico a diversi stadi fenologici. pp. 21-22. in Proc. 12<sup>th</sup>. Nat. Congr. ASPA, Pisa, Italy.
- DOANE, P.H., PELL, A.N., SCHOFIELD, P., 1997. The effect of preservation method on the neutral detergent soluble fraction of forages. *J. Anim. Sci.* 75:1140-1148.
- GEREN, H., SOYA, H., AVCIOÇLU, R., 2003. Investigations on the effect of cutting dates on some quality properties of annual Italian ryegrass and hairy vetch mixtures. *Ege Üniversitesi Ziraat Fakültesi Dergisi* 40(2):17-24.
- GOERING, H.K., VAN SOEST, P.J., 1970. Forage fibre analyses (apparatus, reagents, procedures and some applications). *Agriculture Handbook N. 379*, U.S. Government Printing Office, Washington, DC, USA.
- GROOT, J.C.J., CONE, J.W., WILLIAMS, B.A., DEBERSAQUES, F.M.A., 1996. Multiphasic analysis of gas production kinetics for *in vitro* fermentation of ruminant feedstuffs. *Anim. Feed Sci. Tech.* 64:77-89.
- HADJIPANAYIOTOU, M., ANTONIOU, I., THEODOROU, M., PHOTIOU, A., 1996. *In situ* degradability of forages cut at different stages of growth. *Livest. Prod. Sci.* 45:49-53.
- HOFFMAN, R., DÉR, F., 2004. Yield of different green forage crops, in pure stand and in mixtures. Part 1.: Spring (two rowed) barley (*Hordeum vulgare* L.), pea (*Pisum sativum* L.) and vetch (*Vicia* sp. L.). *Agric. Conspec. Sci.* 68:275-279.
- INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE, 1988. Alimentation des bovines, ovines et caprins. Ed. INRA Publications, Versailles, France.
- LETO, G., ALICATA, M.L., GIACCONE, P., BONANNO, A., 1988. Caratteristiche chimico-nutrizionali del foraggio verde e del fieno di un erbaio di vecchia e avena. *Inform. Agr.* 20:37-39.
- LETO, G., ALICATA, M.L., GIACCONE, P., BONANNO, A., 1989. Caratteristiche nutrizionali del foraggio

- verde di un erbaio di vecchia e orzo. Inform. Agr. 19:33-34.
- MCDONALD, P., EDWARDS R.A., GREENHALGH, J.F.D., 1992. Animal Nutrition. Longman Scientific & Technical, England, UK.
- ØRSKOV, E.R., MCDONALD, I., 1979. The estimation of protein degradability in the rumen from the incubation measurements weighted according to rate of passage. J. Agr. Sci. Camb. 92:499-503.
- PINOSA, M., MAFFEI, M., D'AGARO, E., RIBALDI, E., BERARDI, P., 1995. *Festuca arundinacea*: caratteristiche chimiche e nutritive del foraggio fresco, insilato ed affienato. Zoot. Nutr. Anim. 21:3-12.
- PIÑEIRO, J., SAU, F., CASTRO, P., 2002. Effect of relative seeding rates of oats (*Avena sativa* L.) and common vetch (*Vicia sativa* L.) on total and harvested yield and crude protein content. In: Multifunction grasslands: quality forages, animal products and landscapes. Proc 19<sup>th</sup> Gen. Meet. of European Grassland Federation, La Rochelle, France., pp 466-467.
- ROSS, SM, KING, J.R., O'DONOVAN, J.T., SPANER, D., 2004. Forage potential of intercropping berseem clover with barley, oat, or triticale. Agron. J. 96:1013-1020.
- SAS/STAT® Software 2000. Changes and Enhancements through Release 8.1. SAS Institute Inc, Cary, NC, USA.
- SCHOFIELD, P., PELL, A.N., 1995. Measurement and kinetics analysis of the neutral detergent-soluble carbohydrate fraction of legumes and grasses. J Anim. Sci. 73:3455-3463.
- STEFANON, B., PELL, A.N., SCHOFIELD, P., 1996. Effect of maturity on digestion kinetics of water-soluble and water-insoluble fractions of alfalfa and brome hay. J. Anim. Sci. 74:1104-1115.
- THEODOROU, M.K., 1993. A new laboratory procedure for determining the fermentation kinetics of ruminant feeds. Ciencia. Invest. Agr. 20:332-334.
- THEODOROU, M.K., WILLIAMS, B.A., DHANOA, M.S., MCALLAN, A.B., FRANCE, J., 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Tech. 48:185-197.
- VAN SOEST, P.J., ROBERTSON, J.B., LEWIS, B.A., 1991. Methods for dietary fibre, neutral detergent fibre, and non-starch polysaccharides in relation to animal nutrition. J. Anim. Sci. 74:3583-3597.
- WILLIAMS, B.A., 2000. Cumulative Gas-production Techniques for Forage Evaluation. In: Forage Evaluation in Ruminant Nutrition. CABI Publishing, Oxon, UK, pp 189-214.