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

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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 unfractioned forage and that of its respective NDF. Haymaking decreased gas yield and rate of gas production from the unfractioned 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 FIENAGIONE 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
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 graminae and legumes in pure stand and mixed has been extensively studied (Bittante and Andrighetto, 1982; Casoli et al., 1988; Leto et al., 1988, 1989; Pinosa 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 graminae 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 species 50% of the pure seed (barley: 150 kg ha\(^{-1}\); broad bean: 220 kg ha\(^{-1}\), oats: 140 kg ha\(^{-1}\); vetch: 70 kg ha\(^{-1}\)). The crops received a P and N fertilization of 150 kg P\(_2\)O\(_5\) ha\(^{-1}\) and 60 kg N ha\(^{-1}\), respectively (50% before the seeds and the remaining 50% when the graminae were in the growing phase). Starting from May 2004, forages were harvested at three different growth stages, for grass and legumes, respectively:
end of booting and vegetative growth (1st stage), heading and beginning of flowering (2nd stage), and the beginning of the milky maturation of the grain and pod formation (3rd 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 50µl of heat stable α-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 1. Chemical composition (% DM) of forages.

| Forage               | Stage | DM  | Crude protein | NDF  | NDSC | ADL | ADF  | ADL |
|----------------------|-------|-----|---------------|------|------|-----|------|-----|
| Barley-broad bean    |       |     |               |      |      |     |      |     |
| Fresh 1°             | 16.1  | 17.7 | 48.2          | 20.5 | 8.38 | 26.8 | 4.04 |
| 2°                   | 22.1  | 14.5 | 48.9          | 24.8 | 9.37 | 28.3 | 4.58 |
| 3°                   | 22.8  | 14.0 | 47.8          | 26.9 | 10.2 | 26.5 | 4.86 |
| Hay 1°               | 92.21 | 14.3 | 53.1          | 20.0 | 8.94 | 34.4 | 4.65 |
| 2°                   | 91.45 | 12.1 | 53.8          | 22.1 | 8.83 | 35.2 | 4.75 |
| 3°                   | 91.88 | 11.6 | 55.5          | 19.4 | 11.4 | 34.3 | 6.33 |
| Fresh 1°             | 21.4  | 13.8 | 47.9          | 25.6 | 7.79 | 29.5 | 4.21 |
| 2°                   | 22.9  | 11.3 | 51.1          | 25.8 | 10.5 | 32.8 | 5.38 |
| 3°                   | 25.6  | 10.8 | 52.0          | 27.1 | 10.6 | 35.4 | 5.52 |
| Vetch-oats           |       |     |               |      |      |     |      |     |
| Fresh 1°             | 92.09 | 11.4 | 54.6          | 20.8 | 9.98 | 38.3 | 5.45 |
| 2°                   | 92.30 | 8.21 | 62.5          | 17.9 | 9.14 | 41.9 | 5.71 |
| 3°                   | 93.22 | 9.25 | 59.0          | 21.2 | 10.1 | 42.6 | 5.98 |
| Hay 1°               | -     | 3.04 | 8.84          | 2.43 | 3.67 | 7.01 | 0.51 |
| 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).

ADL: estimated content of NDF fraction (ADL / NDF x 100).

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

The NDF fraction for fermentation studies was prepared according to Van Soest et al. (1991) using α-amylase and sodium sulphite as described previously. The pooled extracted fibre was washed several times with hot distilled water, filtered through a 37 µm 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 ± 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, obtained 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

| Class               | dOM (%) | OMCV (ml/g) | Yield (ml/g) | A (h) | B (h) | C (h) | tmax (h) | Rmax (ml/h) | pH    |
|---------------------|---------|-------------|--------------|-------|------|-------|----------|-------------|-------|
| Barley-broad bean   | 81.8A   | 309         | 380B         | 299A  | 17.1A| 1.52A | 6.03     | 11.1A       | 6.49  |
| Vetch-oats          | 78.0B   | 311         | 396A         | 314A  | 21.6A| 1.40B | 5.87     | 9.36B       | 6.49  |
| 1° stage            | 82.6A   | 319A        | 391A         | 308B  | 17.8B| 1.50  | 5.98     | 11.0A       | 6.51  |
| 2° stage            | 80.1B   | 319A        | 400A         | 314A  | 19.5A| 1.45  | 5.99     | 10.4A       | 6.47  |
| 3° stage            | 77.0C   | 291B        | 374A         | 297B  | 20.7A| 1.42  | 5.89     | 9.32B       | 6.48  |
| Fresh               | 80.1    | 315A        | 392          | 312A  | 17.0B| 1.39B | 4.45B    | 11.6A       | 6.54A |
| Hay                 | 79.7    | 304B        | 385          | 301A  | 21.6A| 1.53A | 7.45A    | 8.82B       | 6.43B |
| 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; tmax: time at which maximum rate was reached. Rmax: 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.
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™, 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 unfractioned 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 unfractioned 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:

\[ G (\text{ml/g}) = \frac{A}{(1+B/\text{time})^c} \]

where \(G (\text{ml/g})\) = 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), \(A (\text{ml/g})\) = asymptotic gas production, \(B (\text{h})\) = time at which \(A/2\) was reached, and \(C\) = switching characteristic of the curve. The maximum rate of gas production \(\left(R_{max}\right)\) and the time at which it occurs \(\left(T_{max}\right)\), were calculated according to following equations (Bauer et al., 2001):
R_{max} (ml/h) = (A - C \cdot B \cdot [T_{max}^{(-B-1)}]/[(1+C \cdot B) \cdot (T_{max}^{B})^{2}])

T_{max} (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.

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, 1st, 2nd and 3rd stage, respectively), whereas barley-broad bean showed a higher ADF content in the 2nd than in the 3rd 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
content was affected by growth stage. Comparing the mean of the two intercropped 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 unfractioned 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); 1st and 2nd stages > 3rd stage (150 and 147 vs 131 ml;...
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 1st, 2nd and 3rd 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 differences 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 (r² = 0.70; P < 0.01) and NDF (r² = 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).

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

| Gas               | A (ml/g) | B (h) | C (h) | t<sub>max</sub> (h) | R<sub>max</sub> (ml/h) |
|-------------------|----------|-------|-------|---------------------|------------------------|
| Barley-broad bean | 148<sup>a</sup> | 155<sup>a</sup> | 8.67<sup>a</sup> | 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<sup>b</sup> | 1.79<sup>b</sup> | 4.13<sup>b</sup> | 10.22<sup>b</sup> |

Forage species effects

| Effect of growth stage | Gas | A (ml/g) | B (h) | C (h) | t<sub>max</sub> (h) | R<sub>max</sub> (ml/h) |
|------------------------|-----|----------|-------|-------|---------------------|------------------------|
| 1<sup>st</sup> stage   | 145 | 149      | 8.13  | 2.17<sup>a</sup> | 4.76<sup>a</sup> | 12.6<sup>a</sup> |
| 2<sup>nd</sup> stage   | 138 | 144      | 8.49  | 1.81<sup>b</sup> | 4.25<sup>b</sup> | 11.1<sup>b</sup> |
| 3<sup>rd</sup> stage   | 139 | 146      | 9.75  | 1.85<sup>b</sup> | 4.74<sup>b</sup> | 9.83<sup>b</sup> |

Forage species effects

| Effect of preservation method | Gas | A (ml/g) | B (h) | C (h) | t<sub>max</sub> (h) | R<sub>max</sub> (ml/h) |
|------------------------------|-----|----------|-------|-------|---------------------|------------------------|
| 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 = mean square error. Means with different small or capital letters in the fraction differ significantly at P < 0.05 or P < 0.01, respectively.

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.

P < 0.01).
very well by the model (R² 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 3rd stage showed at all times lower gas production; the same trend was observed for the fermentation rate compared to the other two stages (Rmax: 12.0 and 11.1 > 9.09 ml/h, for 1st, 2nd and 3rd stage, respectively; P< 0.01).

**Discussion**

**Forage effects**

The two legume-graminæ 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 unfractioned 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 5.** Least squares means representing the main treatment effects for the unfractioned forage for pH and volatile fatty acids.

|                      | Acetate (Mm/g) | Propionate (Mm/g) | Butyrate (Mm/g) | Tot VFA (Mm/g) | A/P | (A+B)/P |
|----------------------|---------------|------------------|----------------|----------------|-----|---------|
| **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** |               |                  |                |                |     |         |
| 1st stage            | 56.46         | 27.57A           | 9.96A          | 93.99A         | 2.06A| 2.438   |
| 2nd stage            | 57.10         | 26.30AB          | 7.99C          | 91.40ABa       | 2.18ABb| 2.49BC  |
| 3rd stage            | 53.52         | 22.64C           | 9.80AB         | 85.97Bb        | 2.39Ab| 2.82A   |
| Fresh                | 57.72A        | 27.09A           | 9.81a          | 94.62A         | 2.15| 2.52    |
| Hay                  | 53.67B        | 23.92B           | 8.70b          | 86.29B         | 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.
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 3rd 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 3rd 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 unfractioned 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 CO2 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, haymaking reduced the maximum fractional rate (Rmax) 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 haymaking may be presented in two ways: 1) unfractioned fresh forage – unfractioned hay; 2) fresh NDS – hay NDS. Assuming that the NDF fraction shows the same behaviour whether isolated or within the unfractioned forage (Schofield and Pell, 1995; Calabrò et al., 2001), the following equations, related to the gas production, can be written:

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

| Class                      | Acetate (Mm/g) | Propionate (Mm/g) | Butyrate (Mm/g) | Tot VFA (Mm/g) | 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>Ab</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.
\[ WF_{\text{fresh}} - WF_{\text{hay}} = (NDF + NDS)_{\text{fresh}} - (NDF + NDS)_{\text{hay}} \quad (1) \]
\[ WF_{\text{fresh}} - WF_{\text{hay}} = NDF_{\text{fresh}} + NDS_{\text{fresh}} - NDF_{\text{hay}} - NDS_{\text{hay}} \quad (2) \]
\[ WF_{\text{fresh}} - WF_{\text{hay}} = NDS_{\text{fresh}} - NDS_{\text{hay}} + (NDF_{\text{fresh}} - NDF_{\text{hay}}) \quad (3) \]

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 unfractioned 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 unfractioned forage and the isolated NDF were similar, as also reported elsewhere (Schofield and Pell, 1995; Stefanon et al., 1996; Calabrò et al., 2001).

The differences in gas production between fresh and hay forages were greater in the 3rd stage for barley-broad bean and in the 2nd stage for vetch-oats, and when there were greater differences between the NDSC contents. Moreover, for the 3rd stage of BB, haymaking also determined a higher reduction of the gas production rate (ml/h) compared to the 1st and 2nd stages (4.9 vs. 1.5 and 2.1 ml/h, respectively). A similar result was observed for the 2nd stage of VO, where storage caused a higher reduction of the fermentation rate compared to the 1st and 3rd 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 faction. 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., 2005). 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 unfractioned 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 (3rd stage) for barley-broad bean and the heading and beginning of flowering (2nd stage) for vetch-oats.

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