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In vitro fermentation patterns and methane production of sainfoin (*Onobrychis viciifolia* Scop.) hay with different condensed tannin contents

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Abstract

Sainfoin (*Onobrychis viciifolia* Scop.) is a perennial legume recently reappraised for some positive characteristics leading to highly satisfactory animal performance. Sainfoin's characteristics may be partly explained by the presence of moderate levels of condensed tannins (CTs) able to protect dietary protein from microbial degradation in the rumen. Decreased CH_4 emissions have been reported for ruminants consuming CT-containing forage. The aim of this study was to evaluate the effects of CT content on the *in vitro* fermentation characteristics and kinetics and methane production of four samples of *O. viciifolia* cut at different phenological stages. Sainfoin hays and one sample of alfalfa hay were incubated at 39°C in anaerobiosis using the *in vitro* gas production technique. The chemical composition, tannin content and fermentation characteristics and kinetics of sainfoin samples were significantly affected by phenological stage. After 48 h, the CH_4 production in sainfoin hays showed a tendency to increase with the advancement of phenological stage [from 38·6 to 49·8 mL g^{-1} of degraded organic matter (OM)]. The best period to cut sainfoin for hay making is between early and late flowering, when the forage combines high OM digestibility, low CH_4 production and more efficient microbial fermentation.

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Introduction

Sainfoin (*Onobrychis viciifolia* Scop.) is a perennial legume that is well adapted to dry hilly environments on calcareous soils, and it is useful for grazing, hay making and silage conservation (Frame *et al.*, 1998). Its use throughout Europe has almost completely ceased during the past 40 years, because of its low persistence and rate of regrowth after the first spring cut (Kallenbach *et al.*, 1996). Recently, sainfoin has been reappraised as a forage crop, because of its positive characteristics leading to highly satisfactory animal performance. Sainfoin has similar crude protein (CP) and digestibility to alfalfa (Koivisto and Lane, 2001) and allows comparable animal performance. It does not induce bloating in ruminants (Howarth *et al.*, 1978) and, in comparison with other forage legumes, it allows a higher absorption of protein in the small intestine (Broderick, 1995).

All these effects may be explained by the presence of moderate amounts of condensed tannins (CTs) in sainfoin (Jones *et al.*, 1976; Koupai-Abyazani *et al.*, 1992). CTs (proanthocyanidins) are polymeric substances composed of flavanol units linked via carbon–carbon bonds that can be found in plant leaves, fruits, wood, bark or roots, sometimes at very high concentrations (Matthews *et al.*, 1997). Because of their distribution within the Leguminosae, these compounds are very often ingested by herbivorous mammals. At high concentrations, they are considered anti-nutritional factors (ANFs), being able to bind dietary proteins selectively, especially those that are large, conformationally open and proline-rich (Hagerman *et al.*, 1992). On the other hand, at low levels, CTs contained in

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forage are able to partially protect dietary protein from microbial degradation in the rumen, increasing the absorption of amino acids from the small intestine (Waghorn, 1996) and improving animal performance in terms of milk production and ovulation rate, while reducing both bloat risks and internal parasite burdens (Min *et al.*, 2003). The amount of tannins may also be affected by harvest time and stages of plant development. Localization and formation of CTs in sainfoin and changes during plant growth have been described elsewhere (Lees *et al.*, 1993, 1995).

From an environmental point of view, feeding ruminants with forage containing CTs may offer potential benefits because of the reduction in urine nitrogen excretion and the depressive effect on rumen methane (CH_4) emission (Waghorn, 2008). Methane emission gives cause for concern because of its contribution to the atmospheric burden of greenhouse gases (GHGs), which are linked to global warming and other related environmental changes (Johnson and Johnson, 1995). Livestock significantly contribute to total farm emissions of GHGs (Steinfeld *et al.*, 2006). A decrease in CH_4 emission has been reported for ruminants consuming CT-containing forage (Patra *et al.*, 2005; Wang *et al.*, 2007) or when a supplemental CT extract was added to a forage-based diet (Carulla *et al.*, 2005).

The aim of this study was to evaluate the effects of tannin content on the *in vitro* fermentation characteristics and kinetics and methane production of *O. viciifolia*. To determine the best stage of development to cut the crop based on nutritional qualities, four different vegetative stages were used.

Materials and methods

Experimental design

Four samples of sainfoin hay and one sample of alfalfa (lucerne; *Medicago sativa* L.) hay were used in this study. Sainfoin samples were collected at four progressive phenological stages, from vegetative to seed pod. Alfalfa hay (AH) was used so that the sainfoin results could be compared with another forage legume commonly used in ruminant nutrition, alfalfa being a species that has a low-tannin content. AH was collected at the flowering stage from a farm in the province of Caserta (Italy). All the forages were analysed for chemical composition and tannin content. The fermentation characteristics, including methane production, were studied using the *in vitro* gas production technique (IVGPT).

Plant material and environmental conditions

Sainfoin samples came from an agronomic trial carried out in 2007–2008 in an upland area of southern Italy (S. Angelo dei Lombardi, Avellino; longitude 40°56′N, latitude 15°10′E, altitude 875 m a.s.l., annual mean temperature 12·6°C and annual average rainfall 758 mm) on a clay-loam soil with a pH of 7·85. The sand, silt and clay contents of the soil were 342, 237 and 421 g kg^{-1}, respectively, at 0–30 cm depth. Organic matter (OM) was 11·5 g kg^{-1}, and organic N was 0·92 g kg^{-1}. Stands of a giant local ecotype of sainfoin called Avellinese were sown in the first 10 d of September at 60 kg ha^{-1} in rows 17 cm apart. At ploughing, the soil was fertilized with 100 kg P_2O_5 ha^{-1}. No irrigation or fertilizers were applied after sowing. All temperature and rainfall data were collected from a weather station located about 200 m from the experimental site.

Herbage samples were collected on randomly located 50-m^2 plots with three replicates cut to a 3–4 cm stubble height. From the beginning of April to the end of June, herbage samples were collected four times at progressive phenological stages from the early vegetative to late seed-pod stage and left in the field until the wilting process was completed. The phenological stage was evaluated on a sample of about 50 stems clipped at ground level and classified according to the 10-stage classification system reported by Borreani *et al.* (2003) in stages 1–4. Accordingly, a pool for each phenological stage was classified as follows: sainfoin hay harvested at early bud stage (SH-1); sainfoin hay harvested at early flowering stage (SH-2); sainfoin hay harvested at late flowering (SH-3); sainfoin hay harvested at early seed pod stage (SH-4).

Chemical composition

All plant samples were ground to pass a 1-mm screen (Brabender Wiley mill, Brabender OHG, Duisburg, Germany) and analysed for dry matter (DM), CP, ether extract (EE) and ash as suggested by AOAC (2000) procedures (ID members: 2001·12, 978·04, 920·39 and 930·05 for DM, CP, EE and ash respectively). Neutral detergent fibre (NDF) was determined by boiling for 1 h a 0·5 g sample in 100 mL of neutral detergent plus 50 μL of heat-stable α-amylase (ANKOM Technology, Macedon, NY, USA) and 0·5 g of sodium sulphite (Van Soest, 1994). Acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined as described by Goering and Van Soest (1970).

Condensed tannins

Condensed tannins in hay samples were analysed by the vanillin–HCl method (Price *et al.*, 1978) with slight modifications. One gram of either sainfoin or alfalfa sample was extracted with 5 mL of technical grade methanol (MetOH) (Sigma-Aldrich, Milan, Italy) using

a reciprocating shaker (1 Hz) for 20 min in 15-mL screw-capped centrifuge tubes. Extractions were performed in triplicate. Finally, the tubes were centrifuged at 1800 ***g*** for 10 min at room temperature. The developing reagent was prepared daily by mixing equal volumes of 5% (w/v) vanillin (Sigma-Aldrich) in MetOH and 8% (v/v) concentrated HCl (Sigma-Aldrich) in MetOH. The reaction mixture was prepared in 15-mL centrifuge tubes by adding 5 mL of the vanillin–HCl reagent to 1 mL of diluted (1/10) methanol extract. After a mixing step, the tubes were incubated for 20 min in a water bath at 30°C and, finally, the absorbance was measured at 500 nm by means of an UV-1601 double-beam spectrophotometer (Shimadzu Corp., Kyoto, Japan). Absorbance measurements were carried out against MetOH by means of 1-cm light-path quartz cuvettes. To take account of other interfering substances in the crude extracts, each sample was also treated as detailed above, incubating 1 mL of diluted samples with 5 mL of the mixture MetOH/8% HCl (1:1). The background was then subtracted from the absorbance value recorded for each vanillin-treated sample. A standard curve was constructed dissolving purified (+)-catechin hydrate (≥96·0%) (Sigma-Aldrich) in MetOH to obtain four calibration standards within the linear regression range ($R^2 > 0{\cdot}99$): 0·125, 0·25, 0·5 and 1·0 mg mL^{-1}. CT content of samples was then expressed in terms of catechin equivalents (CE) as mg CE g^{-1} DM (Price *et al.*, 1978).

Total phenolic compounds

Total polyphenolic compounds were determined according to Zielinski and Kozlowska (2000) with some modifications. Briefly, in 10-mL centrifuge tubes, 0·25 mL of diluted (1:100) methanolic plant extract obtained in the condition described for CT analysis or MetOH (blank) was added to 4·0 mL of ultrapure water and 0·25 mL of water-diluted (1:1) Folin-Ciocalteu reagent (Sigma, Milan, Italy). The mixture was then vortexed for 0·5 min and rested at room temperature for 5 min. A 0·5-mL aliquot of a Na_2CO_3 saturated solution was then added to the mixture and, after mixing, the tubes were incubated at 30°C in the dark for 30 min. After centrifugation at 4200 ***g***, 1 mL of solution was read at 725 nm against blank using a UV-1601 double-beam spectrophotometer (Shimadzu Corp., Japan). A standard curve was constructed dissolving purified (+)-catechin hydrate (≥96·0%) (Sigma-Aldrich) in MetOH to obtain four calibration standards within the range 0·03–0·30 mg mL^{-1}. Total polyphenol content of samples was then expressed in terms of catechin equivalents (CE) as mg CE g^{-1} DM.

In vitro trial

The fermentation characteristics were studied using the *in vitro* gas production technique (IVGPT), by incubating two series of bottles for each sample. To determine methane production, OM degradability and fermentation end products such as ammonia (NH_3) and volatile fatty acids (VFAs), a first series was incubated weighing the samples (1·01 g ± 0·009) in duplicate in 120-mL serum flasks, sealed with butyl rubber and aluminium crimps. The substrate was incubated with 5 mL of rumen fluid and 74 mL of anaerobic medium without nitrogen supply. The rumen fluid was collected in a pre-warmed thermos at the slaughterhouse from six cows fed a standard diet and was then rapidly transported to the laboratory (Calabrò *et al.*, 2009). Two bottles containing no substrate were incubated as blanks to correct for OM disappearance, and gas and end-product production. All the following steps were carried out at 39°C and under insufflations of CO_2 to maintain anaerobic conditions. A detailed description of the equipment used for the gas production measurements is reported by Theodorou *et al.* (1994).

The fermentation was stopped at 48 h, and the fermentation liquor was analysed for pH with a pH meter (Model 3030 Alessandrini Instrument glass electrode; Jenway, Dunmow, UK) and sampled for fermentation end-product analysis. At the end of fermentation, the extent of sample disappearance, expressed as OM digestibility (dOM), was determined by weight difference of the incubated OM and the undegraded filtered (sintered glass crucibles; Schott Duran, Mainz, Germany, porosity # 2) residue following oven-drying to a constant weight at 103°C and burning at 550°C for 5 h. Cumulative volume of gas produced after 48 h of incubation was measured against incubated OM (OMCV, mL g^{-1} iOM).

Gas fermentation kinetics

To study the fermentation kinetics, a second series of bottles (1·01 g ± 0·002 sample weighed) was prepared in triplicate. Gas production of fermenting cultures was recorded nineteen times (at 3–24 h intervals) during the period of fermentation (120 h) using a manual pressure transducer (Cole and Parmer Instrument Co., Vernon Hills, IL, USA). For each flask, the gas production profiles were fitted to the sigmoid mono-phasic model described by Groot *et al.* (1996): G (mL g^{-1}) = $A/(1 + B/t)^C$, where G is the total gas produced (mL iOM^{-1}) at time t (h), A is the asymptotic gas production (mL g^{-1} iOM), B (h) is the time at which one-half of the asymptote is reached, and C is the switching characteristic of the curve. Maximum fermentation rate (R_{max}) and the time at which it occurred

(T_{max}) were calculated according to the following formulas (Bauer *et al.*, 2001): R_{max} (mL h^{-1}) = $(A \cdot C^B) \cdot B \cdot [T_{max}{}^{(-B-1)}] / [(1 + C^B) \cdot (T_{max}{}^{-B})^2]$; T_{max} (h) = $C \cdot [(B - 1) / (B + 1)]^{(1/B)}$.

Methane production

For CH_4 measurement, 3 mL of gas, in duplicate, was sampled from the headspace of each bottle in an airtight syringe at 6, 12, 24 and 48 h of incubation. Methane was determined by injection of sampled gas into a gas chromatograph (GC ThermoElectron® 8000^{top}, CE Instruments, Rodano, Milan, Italy) equipped with a hot wire detector (HWD) and a column packed with Hay-SepQ SUPELCO (3/16 inch, 80/100 mesh). Helium at a flow rate of 30 mL min^{-1} was used as carrier. Oven and detector temperatures were 50 and 190°C respectively.

Fermentation end products

For VFA determination, fermenting liquors were centrifuged at 12 000 ***g*** for 10 min at 4°C (Universal 32R centrifuge; Hettich FurnTech Division DIY, Germany). One millilitre of supernatant was then mixed with 1 mL of oxalic acid (0·06 M). VFAs were measured by gas chromatography (ThermoQuest 8000^{top} Italia SpA, Rodano, Milan, Italy; fused silica capillary column 30 m, 0·25 mm ID, 0·25 μm film thickness), using external standard solution composed of acetic, propionic, butyric, isobutyric, valeric and isovaleric acids, as described by Calabrò *et al.* (2009). Ammonia concentration in the fermentation liquor was analysed by UV spectrophotometer according to Searle (1984).

Statistical analysis

The CTs and total phenolic compounds were statistically analysed (PROC GLM, SAS 2000) to evaluate the differences for these compounds among the five hay samples. The fermentation characteristics (gas, CH_4, NH_3, VFA profile, OM degradability and pH) and the model parameters (A, B, T_{max}, and R_{max}) for the four sainfoin cuts were subjected to analysis of variance (PROC GLM, SAS 2000) to detect the phenological stage effects according to the model: $y_{ij} = \mu + PS_i + \xi_{ij}$, where y is the single datum, μ the mean, PS the phenological stage effect ($i = 4$) and ξ the error term. The comparison between the means was made using the Tukey test. To verify the differences between sainfoin and alfalfa, the orthogonal contrast test was performed (PROC GLM statement CONTRAST, SAS 2000). Moreover, we studied the regression between the chemical composition parameters, including the tannin content, and all the fermentation characteristics (PROC REG, SAS 2000).

Results

Mean annual air temperature and cumulative rainfall during the cropping season from September 2007 to June 2008 (Figure 1) were similar to the seasonal averages previously recorded for the area. During the experimental period, the DM yield was 3·5, 4·0, 4·6 and 5·8 *t* ha^{-1} for SH-1, SH-2, SH-3 and SH-4 respectively.

Chemical composition

The chemical composition of the five hay samples is shown in Table 1. For sainfoin hays, the CP gradually decreased (from 219 to 122 g kg^{-1} DM) as the phenological stages progressed, while the NDF followed the opposite trend (from 391 to 514 g kg^{-1} DM). Compared to sainfoin hay mean values, AH showed higher CP (226 vs. 181 g kg^{-1} DM, for AH and SH respectively), structural carbohydrates (NDF: 523 vs. 445 g kg^{-1} DM, for AH and SH respectively) and ash (109 vs. 66·3 g kg^{-1} DM, for AH and SH respectively) content. The ADL content of AH, albeit lower than that of sainfoin hay samples (mean value: 134 g kg^{-1} DM), was quite high (109 g kg^{-1} DM). Apart for CP, non-structural carbohydrate (NSC) and ash content, AH was more similar to SH-4 than other less mature sainfoin samples (SH-1, SH-2 and SH-3).

Condensed tannins and total phenolics

Methanol extractable CTs of sainfoin, as determined by the vanillin–HCl method, showed the highest value for sample SH-1 (31·5 ± 1·0 mg CE g^{-1} on DM basis) (Figure 2). The level of CTs decreased significantly ($P < 0·01$) in the growth stages that followed (SH-2, SH-3 and SH-4). CTs were not detected in the AH sample. As regards the total phenolic content, a similar trend was observed (Figure 3) to that of CT content, with sample SH-1 having the highest content

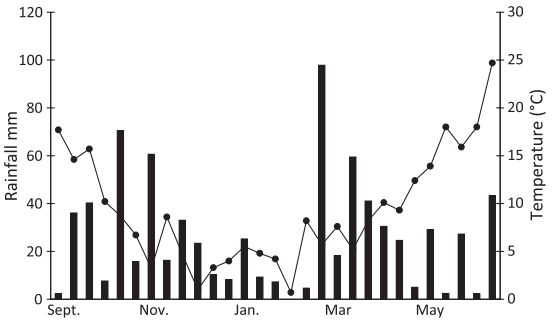

Figure 1 Decadal mean temperature and rainfall during the growth period of sainfoin.

Table 1 Chemical composition of sainfoin hays harvested at different growth stages (sainfoin-1–4) and alfalfa hay (g kg^{-1} dry matter).

Hay	Ash	Crude protein (CP)	Ether extract (EE)	Neutral detergent fibre (NDF)	Acid detergent fibre	Acid detergent lignin	Non-structural carbohydrate (NSC)*
Sainfoin-1	76·6	219	25·7	391	256	166	288
Sainfoin-2	66·5	207	21·9	433	346	136	272
Sainfoin-3	64·8	175	22·3	441	296	119	297
Sainfoin-4	57·4	122	19·0	514	409	113	288
Alfalfa	109	226	13·4	523	457	109	128

Sainfoin-1, hay harvested at early bud stage; sainfoin-2, hay harvested at early flowering stage; sainfoin-3, hay harvested at late flowering stage; sainfoin-4, hay harvested at early seed pod stage.
*NSC (100 – CP – EE – NDF – ash).

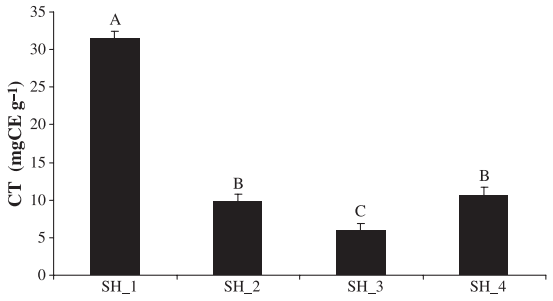

Figure 2 Condensed tannin content of sainfoin at different growth stages. Data (Lsmean ± s.e.) are expressed as mg CE g^{-1} on dry matter basis. SH-1, sainfoin hay harvested at early bud stage; SH-2, sainfoin hay harvested at early flowering stage; SH-3, sainfoin hay harvested at late flowering stage; SH-4, sainfoin hay harvested at early seed pod stage. A, B, C: $P < 0{\cdot}01$.

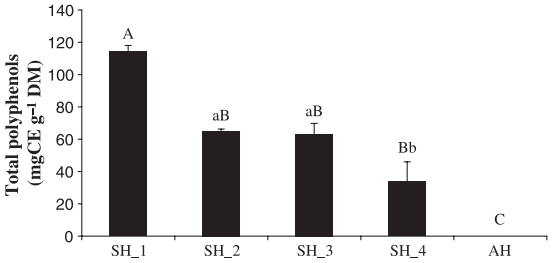

Figure 3 Total polyphenol content of forages. Data (Lsmean ± s.e.) are expressed as mg CE g^{-1} on dry matter basis. SH-1, sainfoin hay harvested at early bud stage; SH-2, sainfoin hay harvested at early flowering stage; SH-3, sainfoin hay harvested at late flowering stage; SH-4, sainfoin hay harvested at early seed pod stage; AH: alfalfa hay. A, B, C, D: $P < 0{\cdot}01$; a, b: $P < 0{\cdot}05$.

(114 ± 5·2 mg CE g^{-1} DM). Compared to sample SH-1, significantly ($P < 0{\cdot}01$) lower levels of total phenolics were observed for the others. No statistical differences were recorded between samples SH-2 and SH-3 (65·1 ± 1·7 and 63·3 ± 9·2 mg CE g^{-1} DM respectively). A very low content of total phenolics was estimated for the AH sample (0·20 ± 0·12 mg CE g^{-1} DM).

Fermentation kinetics

The parameters of fermentation kinetics obtained after 120 h of incubation are reported in Table 2, and the fermentation profiles are illustrated in Figure 4. Comparing the four sainfoin hays, the gas production and fermentation rate profiles appeared similar for SH-2, SH-3 and also for SH-4 until 24 h of incubation, after which time the fermentation process of SH-4 considerably slowed. Both SH-1 and AH fermentation rates were lower than the others, even if the SH-1 fermentation profile improved after 36 h of incubation; T_{max}: 9·56 h). Only the potential gas production and the half-times were statistically different ($P < 0{\cdot}01$) between SH-1 and SH-4 (A: 290 vs. 241 mL g^{-1} and B: 25·3 vs. 19·0 h, for SH-1 and SH-4 respectively). Overall, AH fermented less vigorously, showed lower gas production (A: 236 vs. 271 mL g^{-1}; $P < 0{\cdot}001$) and lower maximum fermentation rate (R_{max}: 6·37 vs. 8·08 mL h^{-1}; $P < 0{\cdot}001$) as well as higher values of B (23·5 vs. 21·4 h; $P < 0{\cdot}05$) and T_{max} (13·6 vs. 10·4 h; $P < 0{\cdot}001$) than sainfoin.

In vitro fermentation characteristics

Some of the fermentation characteristics of sainfoin (dOM, OMCV and NH_3) recorded after 48 h of incubation were significantly ($P < 0{\cdot}05$) affected by the phenological stage (Table 3). As regards the production of total volatile fatty acids (tVFA), acetic, butyric and isovaleric acid (Table 4), the differences among the four

Table 2 *In vitro* kinetics parameters of sainfoin hays harvested at different growth stages (sainfoin-1–4) and alfalfa hay after 120 h of incubation.

	Potential gas production (A) (mL g^{-1} iOM)	Time at which $A/2$ is produced (h)	T_{max} (h)	R_{max} (mL h^{-1})
Hay				
Sainfoin-1	290	25·3	9·56	7·01
Sainfoin-2	276	20·9	10·7	8·34
Sainfoin-3	279	20·3	9·82	8·56
Sainfoin-4	241	19·0	11·6	8·40
Alfalfa	236	23·5	13·6	6·37
Phenological stage effect				
MSD*	38·2	4·163	3·00	2·10
MSD†	27·9	3·04	2·19	1·53
P	0·003	0·001	0·068	0·040
Sainfoin hay vs. alfalfa hay				
MSE	94·1	1·36	0·855	0·293
P	<0·001	0·017	<0·001	<0·001

T_{max}, time at which the maximum fermentation rate is reached; R_{max}, maximum fermentation rate; sainfoin-1, hay harvested at early bud stage; sainfoin-2, hay harvested at early flowering stage; sainfoin-3 hay harvested at late flowering stage; sainfoin-4, hay harvested at early seed pod stage; MSE, mean square error.

*MSD: minimum significant differences for $P < 0·01$.

†MSD: minimum significant differences for $P < 0·05$.

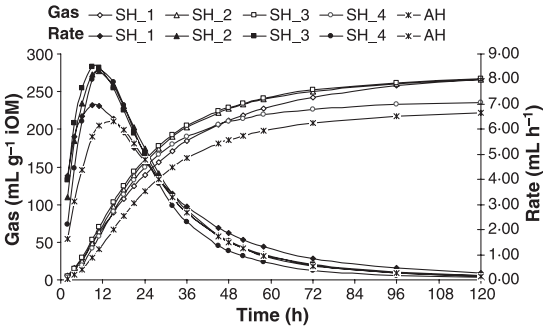

Figure 4 *In vitro* gas production and fermentation rate over time in the forages. SH-1, sainfoin hay harvested at early bud stage; SH-2, sainfoin hay harvested at early flowering stage; SH-3, sainfoin hay harvested at late flowering stage; SH-4, sainfoin hay harvested at early seed pod stage; AH, alfalfa hay.

cuts were highly significant ($P < 0·01$). SH-3 showed higher degradability (dOM: 66·6%), gas (OMCV: 178 mL g^{-1}) and tVFA production (99·7 mM g^{-1} iOM) than the other sainfoin hays. The lowest values were observed in SH-4 for dOM (57·6%) and tVFA (77·5 mM g^{-1} iOM) and in SH-2 for OMCV (147 mL g^{-1}). On average, AH showed significantly ($P < 0·01$) lower OM degradability (51·8 vs. 61·9%), gas (135 vs. 158 mL g^{-1}), tVFA production (76·5 vs. 87·2 mM g^{-1} iOM) and higher NH_3 release (212 vs. 115 mg g^{-1} iOM) than sainfoin.

Methane production

The data obtained for *in vitro* methane production are reported in Table 3 and Figures 5 and 6. During 48 h of incubation, the CH_4 related to both incubated and degraded OM in sainfoin hays showed a tendency, albeit not significant, to increase with the advancement of phenological stage; lower CH_4 production was recorded for AH (20·4 mL g^{-1} iOM and 39·4 mL g^{-1} dOM) with respect to sainfoin (28·2 mL g^{-1} iOM and 45·49 mL g^{-1} dOM) and the difference was significant ($P < 0·01$) when gas was related to incubated OM. Cumulative methane production related to the incubated OM is illustrated in Figure 5. After the first 6 h of fermentation, the trends were clear for all the samples: SH-3 always produced more CH_4 at the highest rate between 12 and 24 h; SH-1 always formed less CH_4 than the rest of the sainfoin samples, with a constant rate (the trend was linear); SH-2 and SH-4 showed at the beginning different rates of CH_4 production that overlapped after 24 h. The AH curve, after a lag phase, overlapped the SH-1 curve within the 18- to 30-h interval. However, for all the forages, the CH_4 profiles appeared to be still growing at the end of observation, meaning that 48 h of incubation was insufficient to complete methane production. Judging from the methane production at four time intervals, expressed as a percentage of the total gas produced (Figure 6), in all samples the amount of CH_4 increased progressively and reached within 24–48 h the maximum of about 30%

Table 3 *In vitro* fermentation characteristics of sainfoin hay harvested at different growth stages (1–4) and alfalfa hay after 48 h of incubation.

	dOM (%)	OMCV (mg g^{-1} iOM)	NH_3 (mg g^{-1} iOM)	CH_4 (mg g^{-1} iOM)	CH_4 (mL g^{-1} dOM)
Hay					
Sainfoin-1	58·1	155	100	22·4	38·6
Sainfoin-2	65·3	147	145	28·3	43·2
Sainfoin-3	66·6	178	122	33·4	50·3
Sainfoin-4	57·6	152	94·8	28·7	49·8
Alfalfa	51·8	135	212	20·4	39·4
Phenological stage effect					
MSD	9·62	27·8	47·5	10·4	30·2
P	0·036	0·036	0·040	0·056	0·164
Sainfoin hay vs. alfalfa hay					
MSE	4·89	41·88	116	5·23	10·8
P	0·002	0·007	<0·001	0·008	0·021

dOM, OM degradability; OMCV, cumulative volume of gas produced related to incubated OM; NH_3, ammonia related to incubated OM; CH_4, methane production related to incubated (iOM) or degraded (dOM) organic matter; sainfoin-1, hay harvested at early bud stage; sainfoin-2, hay harvested at early flowering stage; sainfoin-3, hay harvested at late flowering stage; sainfoin-4, hay harvested at early seed pod stage; MSD, minimum significant differences for $P < 0·05$; MSE, mean square error.

for SH-2, SH-3 and SH-4, 25·4% for SH-1 and 24·1% for AH.

Discussion

Chemical composition

Comparison with other studies (Borreani *et al.*, 2003) with regard to chemical composition is far from straightforward because forage chemical composition varies greatly according not only to harvest stage, but also to soil characteristics, weather conditions and the cultivar utilized. The strong relation between weather conditions and structural carbohydrate content of forages has been observed in many legumes and grass forages (Van Soest, 1994). As expected, sainfoin nutritional characteristics varied according to the vegetative stage; in particular, the increased age at harvest led to a significant decrease in nitrogen content and an increase in cell wall (NDF) content. Surprisingly, the lignin content appeared to decrease during sainfoin growth. The assayability of lignin during the progressive phenological stages may have been influenced by the content of other compounds (e.g. phenols). Indeed, forages containing polyphenolic compounds, such as CTs, may be problematic in the measurement of lignin using the ADF–ADL technique. In forages such as sainfoin and birdsfoot trefoil (*Lotus uliginosus*), Marles *et al.* (2008) reported that the presence of CTs led to an overestimation of the lignin content using conventional gravimetric methods such as the ADL, compared with alternative techniques (e.g. thioglycolic acid lignin assay) that do not account for phenolics. In the light of these findings, taking into consideration that in the SH-1, sample lignin (ADL = 166 mg g DM^{-1}) accounted for more than 42% of the total cell wall matter, the unexpected decreasing pattern showed by lignin in the successive sainfoin samples might be explained in terms of its overestimation in the early growing stages because of the interference of the high phenolic content. In other words, in estimating the lignin content by the ADL method, most probably the observed decrease in polyphenolic content during sainfoin growth did not allow for correct estimation, using the ADF–ADL technique, of the physiological increase in lignin deposition on the plant cell wall. Interestingly, NSC of sainfoin hay were unaffected by the growing stage, and its mean NSC content (286 g kg^{-1}) was more than twice the recorded value for flowering alfalfa (129 g kg^{-1}). Within NSC, sucrose may be the predominant component (up to 35%) of water-soluble carbohydrates (Marais *et al.*, 2000), which could contribute to the high voluntary intake typically described for sainfoin (Karnezos *et al.*, 1994).

Condensed tannins and total phenolic compounds

Condensed tannin content in sainfoin hay was clearly affected by the phenological stage. This is a well-known phenomenon previously described for fruits (Goldstein and Swain, 1963) and also for seeds (Butler, 1982). However, Bullard *et al.* (1981) suggested that a decrease in assayability of CTs in sorghum on maturation is to be

Table 4 pH and volatile fatty acids of sainfoin hay harvested at different growth stages (sainfoin-1–4) and alfalfa hay produced after 48 h of incubation.

	pH	tVFA	Acetic	Propionic	Iso-butyric	Butyric	Iso-valeric	Valeric	ABP	AP	BCP
Hay					mM g^{-1} iOM						
Sainfoin-1	6·35	88·3	58·6	21·3	2·94	3·90	0·99	0·64	2·95	2·76	0·040
Sainfoin-2	6·34	83·3	54·7	17·8	2·76	6·24	1·14	0·68	3·43	3·10	0·041
Sainfoin-3	6·28	99·7	67·5	20·9	2·68	6·47	1·54	0·67	3·54	2·74	0·034
Sainfoin-4	6·32	77·5	49·4	19·7	3·93	4·81	0·76	0·48	2·74	3·81	0·057
Alfalfa	6·44	76·5	49·8	17·8	2·18	4·28	1·68	0·75	3·05	2·81	0·038
Phenological stage effect											
MSD*	0·126	14·7	7·63	6·73	1·66	1·26	0·422	0·645	0·954	3·60	0·016
MSD†	0·079	9·96	5·14	4·53	1·12	0·850	0·285	0·435	0·643	2·42	0·015
P	0·068	0·005	0·001	0·088	0·027	>0·001	>0·001	0·065	0·007	0·011	0·005
Sainfoin hay vs. alfalfa hay											
MSE	$3{\cdot}1^{E-4}$	6·17	1·64	1·28	0·078	0·449	0·005	0·012	0·026	0·365	$1{\cdot}0^{E-4}$
P	<0·001	0·028	>0·001	0·064	0·009	0·001	>0·001	0·176	0·406	0·565	0·925

tVFA, total volatile fatty acids; ABP, (acetate + butyrate) /propionate; AP, acetate /propionate ratio; BCP, brain chain fatty acids proportion (iso-butyric + valeric) /tVFA; sainfoin-1, hay harvested at early bud stage; sainfoin-2, hay harvested at early flowering stage; sainfoin-3 hay harvested at late flowering stage; sainfoin-4, hay harvested at early seed pod stage; MSE, mean square error.
*MSD: minimum significant differences for $P < 0{\cdot}01$.
†MSD: minimum significant differences for $P < 0{\cdot}05$.

explained by increased polymerization. Studies on the typical degree of polymerization of CTs in sainfoin showed that this species has much heavier proanthocyanidins than other forage legumes (Jones *et al.*, 1976). From a merely methodological point of view, both the acidified vanillin and the Folin-Ciocalteu (or Folin-Denis) methods suffer from low stoichiometric yields with respect to the effective quantity of targeted compounds present in the sample's extract (Jansman, 1993). In particular, although the vanillin–HCl method is highly specific for flavan-3-ols, vanillin is known to react more strongly with monomeric flavans (e.g. catechin or gallo-catechin) than with polymeric forms (Salunke *et al.*, 1990). On the other hand, Koupai-Abyazani *et al.* (1993) studying the characteristics of CTs during the growth of sainfoin found only relatively small changes in the mean degree of polymerization of CTs. That said, other causes may be invoked in describing the changes in CT content which we observed in growing sainfoin. From a physiological point of view, a real reduction in CT concentration in growing sainfoin has been shown on studying their synthesis at the leaf level (Singh *et al.*, 1997). Even though the flavan-3-ols biosynthetic pathway was found active in late leaf development stages (Singh *et al.*, 1997) leading to a continuous accumulation of CTs per leaf, a sort of dilution of both extractable and fibre-bound CTs was observed because of plant-cell expansion and growth, giving a decreasing content of CTs when expressed either on a plant fresh-weight or dry-weight basis (Joseph *et al.*, 1998). Looking at all these facts, it seems conceivable that, during development of sainfoin, the decrease in CT concentration we observed in plant tissues really occurs.

Fermentation kinetics

The chosen mono-phasic model (Groot *et al.*, 1996) fits the data of gas production obtained satisfactorily (mean value for $R^2 = 0{\cdot}996$ and for standard error of estimate = 4·50 mL g^{-1}). The fermentation kinetics were affected by chemical composition and /or tannin content. The potential gas production decreased as the phenological stage advanced; these results are consistent with those of Sileshi *et al.* (1996) and Kamalak *et al.* (2005) obtained by incubating several forage types. The reduction in gas production may be a result of decreased microbial activity because of an increased content of cell wall components with advancing plant maturity. However, CP is also one of the limiting factors for microbial growth. Sainfoin harvested at early bud stage, which showed the most favourable chemical composition but also the highest CT and total polyphenol content, evidenced the lowest fermentation rate until 36 h of incubation, after which the fermentation rate exceeded that of the other sainfoin samples. This effect could be related to the tannin level or quality as discussed above and /or to the differential responsiveness to these compounds of different bacteria populations present in the *inocula*. In a comparative study (Jones *et al.*, 1994), purified sainfoin CTs at a concentration around 100 μg mL^{-1} in the culture medium

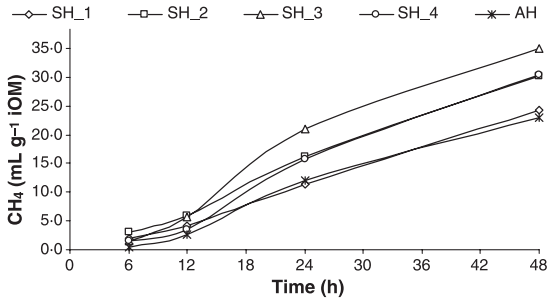

Figure 5 Methane production over time in the forages. SH-1, sainfoin hay harvested at early bud stage; SH-2, sainfoin hay harvested at early flowering stage; SH-3, sainfoin hay harvested at late flowering stage; SH-4, sainfoin hay harvested at early seed pod stage; AH, alfalfa hay.

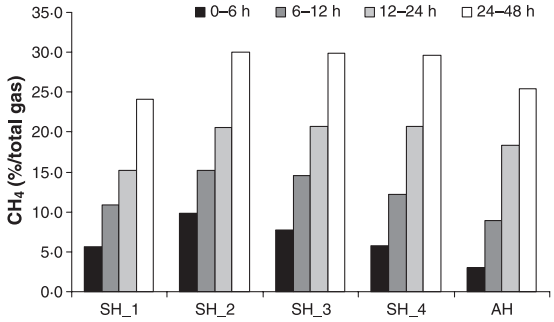

Figure 6 Methane production (% of total gas) at different incubation intervals in the forages. SH-1, sainfoin hay harvested at early bud stage; SH-2, sainfoin hay harvested at early flowering stage; SH-3, sainfoin hay harvested at late flowering stage; SH-4, sainfoin hay harvested at early seed pod stage; AH, alfalfa hay.

were shown to selectively inhibit, albeit to a different extent, the growth and proteolytic activity of *Streptococcus bovis* 45S1, a non-cellulolytic bacterium primarily fermenting starch, and *Butyrivibrio fibrinosolvens* A38, a secondary cellulolytic bacterium. Both microorganisms belong to the Gram-positive group of bacteria, which was supposed to be a possible cause of the strong inhibition exerted by sainfoin CTs. By contrast, this was not observed with two Gram-negative strains of non-cellulolytic rumen bacteria (*Prevotella ruminicola* B_1A and *Ruminobacter amylophilus* WP225) that were able to grow even in a medium containing 600 μg mL^{-1} of CTs. In the light of these findings, incubating sample SH-1 in our conditions simulated a situation in which a nominal concentration of about 400 μg mL^{-1} CTs in the medium was established. It was probably sufficient to exert a strong inhibitory effect on some sensitive bacterial strains, only subsequently replaced by less-sensitive ones through a sort of adaptive process involving several components of the rumen microflora (Kamra, 2005).

The clear effect of tannins on the fermentation kinetics was also confirmed by the effect of polyethylene glycol (PEG), a non-ionic detergent extensively used as a tannin-binding agent (Makkar *et al.*, 1995) that, when added into the fermentation bottle containing the four samples of sainfoin, caused more rapid fermentation in SH-1 and SH-2 compared to SH-3 and SH-4 (our unpublished data). Sainfoin harvested at early seed-pod stage decelerated the fermentation process at 36 h; this trend was probably not related to the tannin content, that was low, but to poor forage quality and limited CP availability. The two intermediate sainfoin samples showed similar chemical composition, tannin content and fermentation kinetics. That said, the evolution of microbial fermentation was regular: when gas produced at 120 h of incubation is measured against degraded OM, the mean value for all the sainfoin hays was 0·375 mL mg^{-1}. This value is very similar to what was found in a previous study (Calabrò *et al.*, 2001) and elsewhere (Schofield and Pell, 1995; Doane *et al.*, 1997): 0·410, 0·390 and 0·350 mL mg^{-1} respectively. Therefore, our results indicate that, despite the presence of at least one class of bioactive compounds (CTs) in incubated samples, fermentation was regular. Thus, it can be asserted that at the level we found, the tannin content of sainfoin did not exert any major toxic effect on rumen microorganisms.

In vitro fermentation characteristics

Organic matter degradability was consistent with that previously reported by Borreani *et al.* (2003), obtained in an analogous *in vitro* study regarding progressive phenological stages of *O. viciifolia*. Despite a linear and significant relationship between gas production, OM degradation and VFA synthesis [r = 0·697 (P < 0·05), 0·701 (P < 0·05) and 0·847 (P < 0·01) for OMCV/dOM, dOM/VFA and OMCV/VFA respectively], some results need more detailed explanations (Table 3 and 4). In comparing SH-2 and SH-3, dOM is similar (65·3 vs. 66·6%), but considerable difference was found in gas (147 vs. 178 mL g^{-1}; P < 0·05), total VFA (83·3 vs. 99·7 mM g^{-1}; P < 0·01) and NH_3 (145 vs. 122 mg g^{-1}) production. Probably, as the regularity of the fermentation process is ensured by the similar trend of gas and VFA, the OM degraded but not fermented (less gas and VFA) for SH-2 may have been used for microbial synthesis (more NH_3). In fact, the degraded OM has a double fate: gas and VFA production and microbial biomass synthesis (Mauricio *et al.*, 2001).

For SH-1, dOM and NH_3 are statistically (P < 0·05) lower than for SH-2 (dOM: 58·1 vs. 65·3%; NH_3: 100 vs.

148 mg g^{-1}). In this case, as reported by some authors (Waghorn, 1996), the high CT content of SH-1 binding the protein (very high in this sample) might have reduced OM degradation. By contrast, the non-significant difference in gas production between SH-1 and SH-2 (OMCV: 155 vs. 147 mL g^{-1}) indicates that carbohydrates have less involvement in this process. Indeed, as reported by McAllister *et al.* (2005), sainfoin is the most desirable source of CTs as it has the highest capacity to bind alfalfa protein and is the least inhibitory to cellulose digestion.

The gas production of SH-1 and SH-4 is comparable (155 vs. 152 mL g^{-1}), but there is a difference in tVFA (88·3 vs. 77·5 mM g^{-1}; $P < 0{\cdot}05$). Although not easy to explain, this result has also been reported by other authors (Getachew *et al.*, 2005).

The lower OM degradability of alfalfa compared to all samples of sainfoin, reported *in vivo* by Aufrére *et al.* (2008), may be because of a lower cell-wall digestibility (Harrison *et al.*, 1973) and a greater leaf loss during drying compared to sainfoin. However, we recognize that the confidence in the differences between sainfoin hays and AH as presented in this paper is limited by use just one alfalfa sample.

pH and ammonia

The pH values after 120 h of incubation for all substrates indicated that the buffering capacity of the medium was always sufficient to maintain the fermentation environment within the 6·2–6·8 range, required to ensure a favourable environment for cellulolytic bacterial activity (Doane *et al.*, 1997).

Ammonia is the most important final product of protein degradation; the values found at the end of fermentation were consistent with the nitrogen content in the forages, except for SH-1 where NH_3 was lower than expected. Protein degradation is likely to have been impeded by the high tannin content which creates a stable complex with proteins, thereby not only protecting them from attack by microorganisms but also possibly interfering with proteolytic activity of bacteria. As reported both *in vivo* (Min and Hart, 2002) and *in vitro* (Jones *et al.*, 1994), CTs are able to reduce the activity of the rumen proteolytic bacteria. CTs can reduce protein degradation mainly through formation of a tannin–protein complex that reduces the possibility of bacterial attack (McSweeney *et al.*, 2001) even if CT reactivity is influenced by molecular weight, the type of tertiary structure and the type of protein amino acids (Min *et al.*, 2003). The inhibitory effect of the tannins, albeit marginal, may also involve fibre digestion (Palmer and McSweeney, 2000). *In vivo* studies with ovines (Barry *et al.*, 1986) demonstrated that the main effect of CTs of *Lotus pedunculatus* at an early vegetative stage was an increase in duodenal flow of non-ammonia nitrogen, hence reduced rumen digestion of hemicellulose and pectin.

Methane production

The study of methane kinetics was useful because the process varies over time. In particular, for the tested forages, the trend is clearly apparent only after 12 h, and more than 48 h of incubation may be needed to complete the process. The mean methane value found in this study for sainfoin (28·2 mL g^{-1} iOM) was slightly lower than those reported by Lovett *et al.* (2006) at 72 h obtained incubating six cultivars of *Lolium perenne*. In this study, sainfoin produced more methane compared to alfalfa (20·4 mL g^{-1} iOM; $P < 0{\cdot}01$). The effects of sainfoin on digestion of alfalfa were studied by McMahon *et al.* (1999); NH_3-N and methane production declined ($P < 0{\cdot}05$) as sainfoin increased. An interesting negative correlation bordering on significance ($r = -0{\cdot}932$; $P = 0{\cdot}068$) was found between the tannin content and CH_4 production, indicating that methane production consistently declined as the CT content increased. As suggested by Carulla *et al.* (2005), inhibition of methanogenesis is because of the reduction in fibre degradation that limits the acetate production through the reduction in cellulolytic number (McSweeney *et al.*, 2001) and/or the poor availability of compounds sequestered by tannins (Makkar *et al.*, 1995). The direct effect of CTs on methanogenic bacteria (Field *et al.*, 1989) should thus be considered. In our case, a clear trend between acetate and methane was not observed.

In SH-1, the lower CH_4 production and concurrently high VFA production indicate that, despite an initial reduction in total gas production, there was no inhibition of the fermentation. Hence, *in vivo* lower methane production, as expected, does not reduce the energy availability for the animal. In the other vegetative stages, the relationship between the CT content and the CH_4 production seemed less direct. The larger amount of methane produced in the fourth sainfoin sample was probably due to the small amount of CTs, but also to the low hay quality found in that late phenological stage. The amount of methane produced is because of a series of interactions between the nutritive characteristics and the presence of ANFs, being both variable with advancing phenological stage. Lovett *et al.* (2006) reported a positive association between CH_4 production and NDF concentrations; in our case, a similar trend was highlighted ($r = 0{\cdot}766$) that does not reach statistical significance because of the lack of available data. The strong relationship between cell wall components and *in vivo* emission of CH_4 has already been discussed by many authors, including Moss *et al.* (2000).

Sainfoin phenological stage and fermentation characteristics

To evaluate the best period to cut sainfoin for hay making, comparing methanogenesis, VFA production and degraded OM may be a very useful approach. The amount of CH_4 and VFAs produced at 48 h was expressed as mmol g^{-1} dOM. Consequently, the CH_4/VFA ratio provides a measure of the amount of CH_4 per unit of VFAs released at 39°C when 1 mg of OM is digested. SH-1 gave the lowest values (0·01016 mmoles of CH_4 per mmoles of VFA) compared to the other sainfoin samples (0·01404, 0·01380 and 0·01514 mmoles of CH_4 per mmoles of VFA, for SH-2, SH-3 and SH-4 respectively) and AH (0·01152 mmoles of CH_4 per mmoles of VFA). As the differences between sainfoin samples were not significant, it would appear that the best period to cut sainfoin for hay making is between early and late flowering: in this period, the forage combines high OM digestibility with low CH_4 production, and potentially not only reduces enteric CH_4 at pasture but also increases ruminant productivity probably due to enhanced levels of microbial biomass production and more efficient microbial fermentation. Also, Borreani *et al.* (2003) found that sainfoin harvested at the early flowering stage will yield forage that is utilized by animals more efficiently in terms of energy gain and low methane emission.

Conclusion

The chemical composition, tannin content, fermentation characteristics and kinetics of *O. viciifolia* were significantly affected by phenological stage. Sainfoin hay harvested between early and late flowering showed good potential to produce high-quality forage. This result is in agreement with good digestibility of OM and good nutritional characteristics, but also the amount of polyphenols, especially CTs, seems to play a major role. Several factors such as cultivar, seasonal variations and soil properties are known to affect the accumulation and structure of tannins. However, the CTs in sainfoin should be regarded as providing real added value for ruminant feeding. The higher methane production from sainfoin than alfalfa should be evaluated overall, taking into account the smaller amount of OM, degraded by livestock and lost to the environment, which contributes to methane production. By suitably choosing the vegetative stage, similar methane emissions may be achieved.

Improving the emissions of methane from ruminants is an area where the agriculture sector can contribute to reducing the global environmental impact of GHG release. Although further studies are needed to explore the use of sainfoin for grazing, hay and silage making in real livestock systems, it appears that sainfoin is a forage species of topical interest and with great potential, given its higher bypass protein content and acceptably low methane emissions.

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