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Alpine pasture plant species affect *in vitro* rumen methane production and kinetics

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ABSTRACT

This study aimed to evaluate the influence of different plant species widespread in alpine pastures on *in vitro* rumen fermentation parameters and methane kinetic production. A total of 11 plant species were sampled at the beginning of the grazing season and used as substrates in an *in vitro* batch fermentation system. After 24h of fermentation, plants affected volatile fatty acids profiles, ammonia yield, and dry matter (DM) digestibilities. *Carum carvi, Ranunculus. acris* and *Festuca rubra* showed the highest total production of methane per unit of digested DM while *Potentilla erecta* was the species that produced less methane. In terms of methane as a percentage of the total gas, *F. rubra* had the highest value (28.9%) while *R. acris* had the lowest (24.2%). Total gas and methane production were monitored continuously and the percentage of methane in total gas was fitted with the Gompertz model. Plants differed significantly ($p < .01$) in methane production kinetics, including production rate decline (A), asymptotic methane concentration (B), time to maximum fermentation rate (TMFR), and maximum fermentation rate (MFR). *C. carvi*, *Prunella grandiflora*, and *R. acris* showed high values of MFR and the top values in the production rate decline ($A > 0.9$). The two grasses (*F. rubra* and *Poa alpina*) together with *Hypericum maculatum* showed an opposite behaviour with low values in MFR, A and a longer TMFR. The results of the methane production kinetics allow for an in-depth evaluation of plant species, adding further information to those registered at the end of fermentation.

HIGHLIGHTS

- Plants were evaluated by end-point fermentative traits and by 24-hour fermentation methane production kinetics.
- *F. rubra* had the highest methane yield as a percentage of total gas while *R. acris* the lowest (28.9% and 24.2%, respectively)
- Despite total methane production, plants differed significantly in their methane kinetics.

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Introduction

The ability of ruminants to transform fibrous and low-quality nitrogen compounds into highly nutritive foods with limited competition with human resources is mainly due to the fermentations that occur in the rumen. However, ruminal fermentation generates also methane (CH_4), which represents a significant inefficiency in feed utilisation with gross energy loss of around 2 to 12% (Johnson and Johnson 1995). Moreover, it contributes 3.3% to the total greenhouse gas emissions and 17% to the global CH_4 sources (Knapp et al. 2014).

Because enteric methane represents an important input to environmental pollution and climate change, research is focused on the study of the different factors that influence its production to identify approaches for its reduction (Hristov et al. 2013; Beauchemin et al. 2020). Over the years, various strategies have been developed to address this issue, including dietary management (Arndt et al. 2022).

Pastures are key feed supplies for farm ruminants and have also important implications in terms of social and environmental issues in some territorial contexts, such as the alpine areas (Morgan-Davies et al. 2014). In addition, among the grazed plants, there are many species rich in secondary metabolites (Jayanegara et al. 2011; Nardin et al. 2023) which may differently affect rumen methanogenesis depending on the type (e.g. alkaloids, phenols, or tannins) and their concentration in the diet (Niderkorn and Baumont 2009; Ku-Vera et al. 2020).

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Some *in vivo* methods for methane assessment could be used in pasture conditions, but they can alter animal feed intake, behaviour and overall production traits (Thompson and Rowntree 2020). *In vitro* gas production systems are a useful alternative tool for research on methane emissions (Yáñez-Ruiz et al. 2016). These techniques are widely used to rapidly detect methane resulting from the ruminal fermentation of several substrates, including plant species (Tavendale et al. 2005), or additives such as plant extracts (Manoni et al. 2023). The main aim of this study is to evaluate the effect of different plant species present in the Alpine pastures on methane production and ruminal fermentation parameters. Furthermore, through the application of an *in vitro* system for continuous measurement, the methane yield kinetics will be analysed.

Materials and methods

Plant sampling

According to previous outcomes from studies conducted on alpine pastures in northeastern Italy (Gianelle et al. 2017; Romanzin et al. 2018; Nardin et al. 2023) and co-operating with a botanist expert in local flora and animal-plant relationships, 11 forage species were selected for the present study (*Achillea millefolium L., Carum carvi L., Festuca rubra L., Hypericum maculatum Crantz subsp. maculatum, Lotus corniculatus L., Poa alpina L. subsp. alpina, Potentilla erecta L. Raeusch., Prunella grandiflora L. Scholler, Ranunculus acris L., Trifolium repens L.*, and *Veronica chamaedrys L.*). Furthermore, plant selection was driven by the contribution that these plants have to grazing dairy cows' diets, considering their availability and palatability. All alpine forage plant species were sampled in the Malga Montasio pastures (Chiusaforte, Italy; 46°24′45″N, 13°25′53″E; 1500–1800 m above sea level) at the end of June 2023. Samples were collected at the beginning of the grazing season before the cows entered the pasture at three different sites (*Poion alpinae* Alliance, Bovolenta et al. 2014). The whole plant was cut about 3 cm above the ground, and approximately 0.5 kg of fresh matter was collected for each. Then samples were vacuum packed, stored at refrigeration temperature (4 °C) and transported to the laboratory in controlled conditions.

Plant analysis and in vitro experiment

Each plant sample was divided into two sub-samples. The first one was used for chemical composition analysis. Plants were dried (60 °C for 48 h), milled through a 1 mm screen (Ciclotec Tecator), and analysed for dry matter (DM), crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) (Van Soest et al. 1991), ash, and ether extract (EE) following the Association of Official Analytical Chemists protocol (AOAC 2000). The second sub-sample was used as a fresh substrate for the *in vitro* fermentation. Plants have not been heated to preserve the bioactive compounds naturally present in plants, maintaining their characteristics (ElGamal et al. 2023). Substrates were roughly cut (to about 1 cm) and smashed manually to simulate the cows' chewing and then kept frozen (–20 °C) until their utilisation in the fermentation system. After being thawed, samples were used as wet substrates in an *in vitro* rumen fermentation trial, being careful to ensure the same level of DM (3300 mg) in all fermenters. The *in vitro* equipment layout was previously reported by Braidot et al. (2023a). Briefly, the apparatus was composed of six glass fermenters with an available volume of 750 mL plugged into a gas counter (Ritter Apparatebau GmbH & Co. KG) that allows the gas production measurement. The counter outflow was connected to an infra-red gas analyser sensor (RI. sens mono IR1, Ritter Apparatebau GmbH & Co. KG) for continuous monitoring of methane concentration. Each fermentation bottle was filled with 500 mL of a mix composed of filtered rumen fluid and the buffer solution proposed by Menke et al. (1979) (ratio 1:2, v/v). The rumen fluid for each fermentation run was collected in the same slaughterhouse from 4 culled dairy cows, slaughtered in good health conditions for production purposes and previously fed total mixed rations based on corn silage. The fermenters were incubated 24 h at 39 °C and during incubation gas and methane production were monitored continuously. In each fermentation run, a fermenter with the buffered rumen fluid without any substrate was incubated to monitor gas and methane net production and data were used as 'blank' values.

Analysis of fermentation fluid

After 24 h of incubation, the pH was directly measured using a pH metre (GLP 22, Crison Instruments, S.A. Barcelona, Spain), and then samples of fermentation fluid were collected for ammonia, VFA profile, and protozoa determination. The ammonia concentration was quantified using an Ammonia Gas Sensing Combination Electrode (Hach Company, Colorado, USA) following the manufactory protocol. The VFA

concentration was assessed as described in detail by Spanghero et al. (2023). Briefly, 5 mL of fermentation fluid was acidified by adding 5 mL of H_2SO_4 0.1 N (ratio 1:1 v/v) and then centrifuged at 20,000 *g* for 20 min at 4 °C. Subsequently, the supernatant was filtered using polypropylene filters (pore diameter 0.45 μm) and transferred into autosampler vials. The VFAs were determined using high-performance liquid chromatography. Finally, for the protozoa quantification, 5 mL fermentation fluid was diluted with 5 mL of 18.5% formaldehyde solution (1:2 ratio, v/v), and then the sample was transferred into a counting chamber and protozoa counted using an optical microscope as described by Dehority (2003). The dry matter disappearance (DMD) was calculated by assessing the residue DM after fermentation. The solid fraction was recovered by centrifugation of the whole content of each fermenter at 4600 *g* for 5 min. The pellet was washed with distilled water and subsequently dried at 60 °C for 48 h. After residual DM determination, the DMD was estimated as follows:

$$DMD(\%) = \left(1 - \frac{g\ Residue\ DM - g\ Blank\ DM}{g\ incubated\ DM}\right) * 100$$

Experimental design and statistical analysis

Given the limited number of bottles in the *in vitro* system, it was not possible to test all plants in the same *in vitro* fermentation run. Therefore, a balanced incomplete block design arrangement was used, as reported by Cox and Reid (2000). A total of eleven fermentation runs (blocks) were performed and each plant was tested in five distinct fermentation runs to allow all pairs of plants to occur together within a block an equal number of times ($\lambda = 2$).

The percentage of methane in total gas recorded each hour from each fermentation bottle was fitted with the Gompertz model:

$$Y_t = B(\exp^{(-Cexp^{-At})})$$

where Y_t is the CH_4 produced (% in total gas) at a specific time (t), B is the asymptotic methane volume (%), C is the specific CH_4 production rate (1/h) dependent on time (t), and A is a constant that describes the decline of the production rate. The lag phase (Lag), the maximum fermentation rate (MFR), and the time to maximum fermentation rate (TMFR) have been calculated using the parameters of the Gompertz equation as described by Lavrenčič et al. (2015).

Data collected from the *in vitro* trials (fermentative traits, gas and methane yields and methane kinetics parameters) were statistically analysed by the GLM procedure of SAS software (Version 9.4; SAS Institute) and the following factorial model:

$$Y_{ij} = \mu + a_i + \beta_j + \varepsilon_{ij}$$

where Y_{ij} is the outcome for the ith plant in the jth fermentation run, μ is the overall mean, α_i is the fixed effect of the plant ($i = 1,11$), β_j is the random effect of the fermentation run ($j = 1,11$), and ε_{ij} is the residual error.

The principal component analysis (PCA) was realised using the PRINCOMP procedure in SAS. The proximal composition, the fermentative traits, and the kinetic parameters were included in the analysis. Only the components with eigenvalues greater than 1 were retained, and to calculate the factor scores of each plant, only the first two PCs were considered.

Results

The chemical composition of plants is reported in Table 1. The DM content ranges from the lower values of *C. carvi* and *P. grandiflora* (18.5% and 20.5%, respectively) to the higher values of *F. rubra* and *P. alpina* (40.3% and 43.7%, respectively). These two plants showed also the highest levels of NDF and ADF. The highest CP content was observed in *T. repens* (24.6% DM) and *L. corniculatus* (20.3% DM) while the two grasses, *F. rubra* and *P. alpine*, registered the lowest values (7.59% DM and 6.92% DM respectively).

The pH, ammonia, DMD, and protozoal count measured in the fermentation fluid after 24 h of fermentation are shown in Table 2. A significant effect of plants ($p < .01$) was observed for ammonia concentration, with values ranging between the highest of *T. repens* (65.5 mg/dL) and the lowest of *P. grandiflora* (42.5 mg/dL). Plants had also a significant effect on total DMD ($p < .01$), with *T. repens* showing the highest

Table 1. Proximal composition of plants used in the *in vitro* trial.

		Composition (% DM)					
	DM	CP	NDF	ADF	ADL	Ash	EE
Achillea millefolium	25.7	16.8	49.2	26.4	8.06	7.73	2.02
Carum carvi	18.5	16.1	41.9	24.5	6.15	9.29	1.72
Festuca rubra	40.3	7.59	74.8	40.7	5.77	2.53	1.50
Hypericum maculatum	31.7	11.5	56.2	38.4	21.7	5.57	1.61
Lotus corniculatus	22.5	20.3	42.6	24.9	10.4	7.37	1.92
Poa alpina	43.7	6.92	69.1	34.7	4.48	2.78	1.96
Potentilla erecta	32.2	12.0	51.6	28.8	8.70	5.20	1.41
Prunella grandiflora	20.5	10.6	42.8	24.0	10.2	8.61	1.22
Ranunculus acris	23.0	8.90	51.7	32.1	8.00	5.83	2.18
Trifolium repens	23.0	24.6	35.9	19.9	6.16	10.7	1.85
Veronica chamaedrys	28.0	11.4	44.5	27.3	11.7	6.28	0.76

DM: dry matter; CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; EE: Ether extract.

value (75.2%) and *F. rubra* showing the lowest (32.8%). No statistically significant changes were observed in the Entodinia and Holotrica groups of protozoa.

Table 3 shows the total concentration and the profile of VFA assessed in the fermentation fluid after incubation. Between substrates, similar values in total VFA production were observed ($p > .05$) while significant variations ($p < .01$) were registered in the proportion of individual VFA analysed. Particularly, acetate (C2) ranged from 69.2% to 74.4%, propionate (C3) from 11.2% to 14.0%, butyrate (C4) from 9.43% to 13.0%, and valerate (C5) between 0.49% and 0.98%. *H. maculatum* had the highest value for C2 (74.4%), the lowest value for C3 (11.2%), and consequently the highest C2:C3 ratio among all plants examined (6.75). Moreover, this plant showed the lowest value in C5 (0.49%). *P. alpina* presented the opposite situation, with the lowest values for C2 (69.2%) and the highest values for C3 (69.2 and 14.0%, respectively), C5 (0.98%), and iso-valerate (Iso-C5, 2.71%). This plant had the lowest C2:C3 (4.93), but similar ratio values were observed in *F. rubra, C. carvi,* and *L. corniculatus*. The highest C4 (13.0%) and lowest iso-butyrate (Iso-C4, 0.57%) values were reached when *P. grandiflora* was used as substrate. The lowest value for C4 (9.43%) was detected in *T. repens*.

As regards fermentation gases (Table 4), there was a significant effect of plants on total gas (mL/g DMD, $p < .01$), methane production (mL/g DMD, $p < .01$), and methane as a percentage of total gas ($p < .05$). Total gas reached the highest production for *C. carvi* and for *R. acris* (523 and 513, respectively), while *P. erecta* was less fermentable than other plants tested with the lowest gas value registered (191).

The ranking of *C. carvi, R. acris*, and *P. erecta* for total gas was also observed for total methane yields

Table 2. LS means of fermentative traits obtained after 24 h of *in vitro* incubation.

	pH	NH_3 (mg/dL)	DMD (%)	Protozoa (10^3 Cell/mL) Entodinia	Holotrica	Total
Achillea millefolium	6.80	60.9^{ab}	52.9^{abcd}	155	15.9	171
Carum carvi	6.79	55.4^{abc}	48.5^{bcd}	182	29.9	212
Festuca rubra	6.81	55.6^{abc}	32.8^{d}	178	29.1	208
Hypericum maculatum	7.03	53.1^{abc}	36.3^{cd}	158	30.1	189
Lotus corniculatus	6.73	56.5^{ab}	65.3^{ab}	162	19.7	182
Poa alpina	6.81	60.2^{ab}	41.7^{bcd}	160	32.4	193
Potentilla erecta	6.83	50.4^{bc}	61.4^{ab}	187	25.3	212
Prunella grandiflora	6.73	42.5^{c}	60.2^{ab}	205	23.5	229
Ranunculus acris	6.76	55.0^{abc}	41.6^{bcd}	148	24.6	173
Trifolium repens	6.79	65.5^{a}	75.2^{a}	171	18.0	189
Veronica chamaedrys	6.79	50.3^{bc}	57.7^{abcd}	165	15.1	180
Significance[a]	NS	**	**	NS	NS	NS
RMSE	0.144	5.98	10.7	24.7	9.34	27.6

DMD: dry matter disappearance; RMSE: Root means square error.
Within columns, means with different superscripts (^{a,b,c,d}) are diverse ($p < .05$).
[a]Overall effect of substrate ($p < .01$).

Table 4. LS Means of gas and methane measured after 24 h of *in vitro* fermentation.

	Gas (mL/g DMD)	Methane (mL/g DMD)	Methane (%)
Achillea millefolium	432^{ab}	106^{ab}	27.8^{ab}
Carum carvi	523^{a}	126^{a}	24.5^{ab}
Festuca rubra	398^{abc}	112^{a}	28.9^{a}
Hypericum maculatum	254^{bc}	64.6^{ab}	25.3^{ab}
Lotus corniculatus	319^{abc}	80.7^{ab}	24.9^{ab}
Poa alpina	339^{abc}	89.2^{ab}	26.7^{ab}
Potentilla erecta	191^{c}	49.2^{b}	24.6^{ab}
Prunella grandiflora	303^{abc}	79.8^{ab}	26.6^{ab}
Ranunculus acris	513^{a}	122^{a}	24.2^{b}
Trifolium repens	253^{bc}	65.3^{ab}	26.6^{ab}
Veronica chamaedrys	312^{abc}	73.7^{ab}	24.3^{ab}
Significance[a]	**	**	*
RMSE	103	26.8	1.99

DMD: digested dry matter; RMSE: Root means square error.
Within columns, means with different superscripts (^{a,b,c}) are diverse ($p < .05$).
[a]Overall effect of substrate $p < .01$ (**) or $p < .05$ (*).

Table 3. LS Means of total volatile fatty acids and their profile obtained after 24 h of *in vitro* incubation.

		% Total VFA						
	Total VFA (mM)	C2	C3	C4	Iso-C4	C5	Iso-C5	C2:C3
Achillea millefolium	36.4	71.4^{abc}	12.9^{abc}	11.3^{ab}	0.80^{abc}	0.94^{a}	2.60^{a}	5.57^{ab}
Carum carvi	36.3	72.0^{abc}	13.7^{ab}	10.3^{ab}	0.80^{abc}	0.70^{bc}	2.51^{ab}	5.36^{b}
Festuca rubra	38.6	70.0^{bc}	13.2^{ab}	12.3^{ab}	0.89^{a}	0.92^{a}	2.67^{a}	5.37^{b}
Hypericum maculatum	26.3	74.4^{a}	11.2^{c}	10.5^{ab}	0.70^{abc}	0.49^{c}	2.65^{a}	6.75^{a}
Lotus corniculatus	29.5	70.8^{abc}	13.5^{ab}	12.1^{ab}	0.66^{abc}	0.65^{bc}	2.25^{ab}	5.34^{b}
Poa alpina	34.6	69.2^{c}	14.0^{a}	12.3^{ab}	0.88^{ab}	0.98^{a}	2.71^{a}	4.93^{b}
Potentilla erecta	32.8	72.6^{abc}	12.0^{bc}	11.3^{ab}	0.73^{abc}	0.71^{b}	2.64^{a}	6.10^{ab}
Prunella grandiflora	28.4	71.6^{abc}	12.2^{abc}	13.0^{a}	0.57^{c}	0.67^{bc}	1.95^{c}	5.97^{ab}
Ranunculus acris	34.8	73.3^{ab}	12.3^{abc}	10.6^{ab}	0.67^{abc}	0.96^{a}	2.15^{ab}	5.97^{ab}
Trifolium repens	50.4	73.9^{a}	12.6^{abc}	9.43^{b}	0.84^{abc}	0.65^{bc}	2.59^{a}	6.17^{ab}
Veronica chamaedrys	35.9	71.8^{abc}	13.1^{bc}	11.3^{ab}	0.62^{bc}	0.82^{ab}	2.27^{ab}	5.48^{ab}
Significance[a]	NS	**	**	**	**	**	**	**
RMSE	11.5	1.57	0.77	1.33	0.11	0.26	0.09	0.56

C2: acetate; C3: proprionate; C4: butyrate; Iso-C4: iso-butyrate; C5: valerate; Iso-C5: iso-valerate; C2:C3: acetate propionate proportion; RMSE: Root means square error.
Within columns, means with different superscripts (^{a,b,c}) are diverse ($p < .05$).
[a]Overall effect of substrate ($p < .01$).

(125, 122, and 49.2, respectively). Moreover, *F.rubra* was the plant with the highest volume of methane as a percentage of the total gas produced (28.9) while *R. acris* showed the lowest production (24.2).

Table 5 shows the kinetic parameters of the methane production expressed as a percentage of total gas. As reported, plants significantly affected ($p < .01$) the decline of production rate (A), asymptotic methane concentration (B), time to maximum fermentation rate (TMFR), and maximum fermentation rate (MFR). *C. carvi* was the plant with the greatest decline in methane production rate (1.02) while grasses (*F. rubra* and *P. alpina*) and *P. erecta* had lower rates (from 0.37 to 0.43). *P. grandiflora* had asymptotic methane concentration values greater than 28%, while *H. maculatum*, *V. chamaedrys*, *R. acris*, *C. carvi*, and *L. corniculatus* exhibited values lower than 25%. Analysing TMFR, similar outcomes were reached for *C. carvi* and *R. acris* (1.87 h and 1.88 h, respectively), while *F. rubra* revealed the highest value (3.57 h). In contrast, *F. rubra* showed lower MFR (4.00%/h), similar to *P. erecta* (3.51%/h), while *C. carvi* (8.88%/h) and *P. grandiflora* (9.30%/h) had the highest values. Despite the wide range of lag values (from 0.20 to 0.90 h) the variations were not statistically significant.

Table 5. Kinetic parameters of methane percentage calculated using the Gompertz model for each plant tested.

	A	B, %	C, 1/h	TMFR, h	MFR, %/h	Lag, h
Achillea millefolium	0.82^{bcd}	25.0^{bc}	4.57	2.22^{cde}	7.62^{ab}	0.72
Carum carvi	1.02^{a}	24.5^{c}	4.87	1.87^{e}	8.88^{a}	0.60
Festuca rubra	0.38^{f}	29.1^{a}	4.07	3.57^{a}	4.00^{d}	0.84
Hypericum maculatum	0.49^{ef}	23.9^{c}	2.98	2.64^{bc}	4.58^{cd}	0.37
Lotus corniculatus	0.75^{cd}	24.9^{c}	4.73	2.26^{cde}	6.89^{abc}	0.73
Poa alpina	0.43^{f}	28.8^{a}	4.30	2.88^{b}	4.89^{cd}	0.73
Potentilla erecta	0.37^{f}	26.1^{abc}	2.62	2.31^{bcde}	3.51^{d}	0.20
Prunella grandiflora	0.91^{abc}	28.3^{a}	5.65	2.43^{bcde}	9.30^{a}	0.90
Ranunculus acris	0.95^{ab}	24.2^{c}	4.87	1.88^{e}	8.27^{ab}	0.67
Trifolium repens	0.63^{de}	27.8^{ab}	4.24	2.46^{bcd}	6.49^{bc}	0.76
Veronica chamaedrys	0.87^{abc}	24.1^{c}	5.61	1.99^{de}	7.91^{ab}	0.61
Significance[1]	**	**	NS	**	**	NS
RMSE	0.12	1.73	1.10	0.36	1.40	0.28

Abbreviations: A: decline of production rate; B: asymptotic methane; C: specific CH4 production rate; TMFR: time to maximum fermentation rate; MFR: maximum fermentation rate; RMSE: Root means square error.
Within columns, means with different superscripts (^{a,b,c,d,e,f}) are diverse ($p < .05$);.
[1]Overall effect of substrate ($p < .01$);.

The plot of the first two principal components (PC) describing the relationships among plant chemical components and *in vitro* ruminal fermentation traits are shown in Figure 1 (plot a). A close relationship between plant composition and *in vitro* ruminal fermentation variables was found by PC analysis, with the first PC explaining 27.2% and the second 22.1% of the variability. The first component showed a positive correlation between CP, DMD, gas, and MFR, but a negative correlation with NDF and TMFR. CP, DMD, gas, and MFR were positively correlated with each other on the first component, but they were negatively correlated with NDF and TMFR. C2 was negatively correlated, on the second component, to C3. In the principal axis, the legumes, *C. carvi* and *A. millefolium* are on the right against *H. maculatum* and grasses. While on the secondary axis, the grasses are opposite *P. grandiflora* and *H. maculatum*. *T. repens*, *L. corniculatus*, *A. millefolium*, and *C. carvi* were positively associated with CP, DMD, gas, MFR, and with the decline in methane production rate. Finally, *F. rubra* and *P. alpina* were positively associated with NDF.

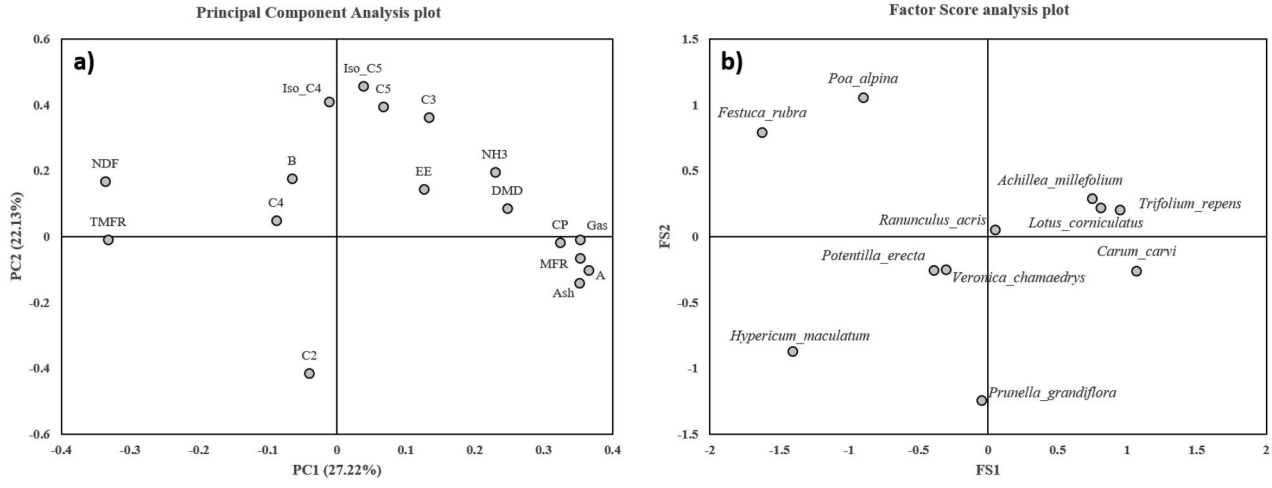

Figure 1. The plot of the first two PC loadings (a), describing the relationship among plant composition and *in vitro* ruminal fermentation and the plot of the first two factor scores (b), describing the classification of each plant within the PC loading.
CP: crude protein; EE: ether extract; NDF: neutral detergent fibre; DMD: dry matter disappearance; C2: acetate; C3: propionate; C4: butyrate; C5: valerate; Iso-C4: iso-butyrate; iso-C5: iso-valerate; A: decline of production rate; B: asymptotic methane; TMFR: time to maximum fermentation rate; MFR: maximum fermentation rate.

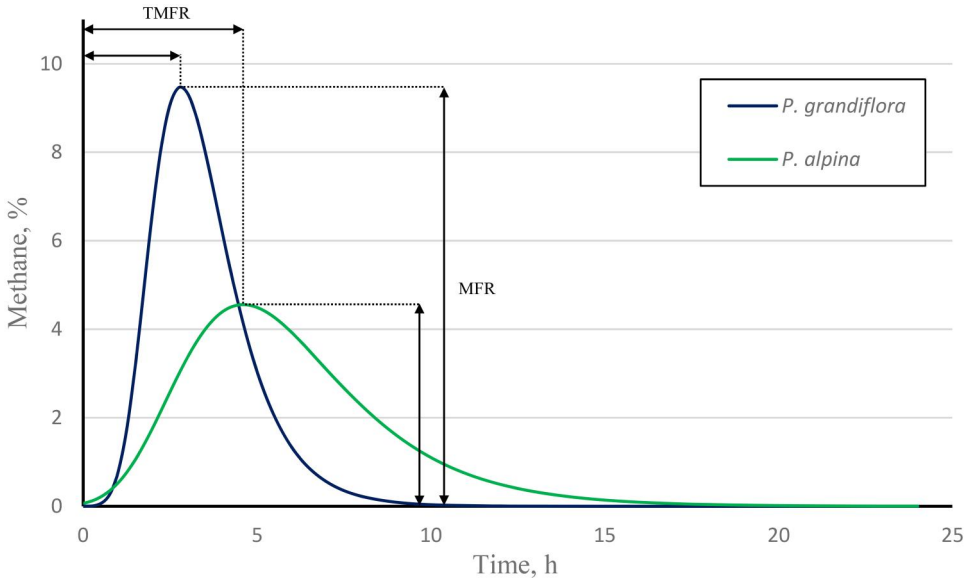

Figure 2. The first derivative of the Gompertz model calculated for *P. alpina* and *P. grandiflora* considering the lag phase. TMFR: time to maximum fermentation rate; MFR: maximum fermentation rate.

Discussion

Plants synthesise primary organic compounds (such as fibres, starch, protein, etc.) that are needed for structural function, reproduction needs, storage, and metabolic purposes. Thereafter, they also produce other organic compounds, and secondary metabolites (e.g. tannins, saponins, essential oils, etc), which are typically synthesised in small amounts and often ensure sensorial properties (colour and taste) and/or protection against biological attacks (e.g. pathogen microorganisms, insects and animal predators). In a natural environment, such as a pasture, ruminants utilise large amounts of different plant essences, which supply nutrients and also substances that can impact rumen microbial activity

Chemical composition and in vitro rumen fermentation parameters

Plants in this study were sampled during the phenological phase of flowering, when they are generally consumed by animals on alpine pastures, except for two grasses (*F. rubra* and *P. alpina*), which were in the phenological stage of fruiting. The high values in DM, NDF, and ADF content detected in plants confirm the advanced phenological stage. The legumes used in the present study (*L. corniculatus* and *T. repens*) had limited vegetative development, in terms of height, but, generally, they are widespread in pastures with high vegetative cover. These plants are characterised by low NDF content, high digestibility, and the highest amount of CP. Furthermore, they are highly palatable to cattle and thus provide an important contribution to meeting protein needs (Mora-Ortiz and Smith 2018). Given their chemical composition, legumes are positively correlated to CP and negatively correlated to NDF as can also be seen in Figure 1. The main fermentative traits were affected by plants' proximal composition. The CP content influences ammonia production, with amino acids deriving from feed proteins that could be used by microbial metabolism or deaminated into ammonia. As can be observed in Table 2, *T. repens* has the highest CP content and also the highest ammonia concentration. In contrast, *L. corniculatus* has reached a lower ammonia value despite its high CP content and this phenomenon is probably due to the presence of tannins that might affect protein availability, as reported by Piluzza et al. (2014). This compound has been demonstrated to affect the ammonia concentration given its capacity to create a tannin-protein complex at ruminal pH, reducing protein degradability (Jayanegara et al. 2020), methane production (Jayanegara et al. 2012; Piluzza et al. 2014) and microbial development (Bhatta et al. 2009; Vasta et al. 2009; Jayanegara et al. 2015; Aboagye and Beauchemin 2019). The DMD was related to the chemical composition of plants, with a higher value in DMD reached with *T. repens* (75.2%), characterised by the lowest NDF and ADF content, while *F. rubra* has a lower DMD value of 32.8% given the higher NDF and ADF percentage. The chemical composition of plants influences both the DMD and the main fermentative traits. The highest DMD achieved by *T. repens* is due to its low NDF and ADF contents, while the opposite situation was observed in *F. rubra* characterised by the lowest DMD but high NDF and ADF content. Different

DMD influence the total VFA produced with *T. repens* and *F. rubra* featured by the highest and lowest values at the end of fermentation. Plants used have modified significantly the VFA profile: *H. maculatum* and *P. alpina* had comparable contents of NDF and ADF but showed a very different level of lignification. In particular, *H. maculatum* showed the highest ADL and consequently, a limited amount of hemicellulose while the opposite was found in *P. alpina*. Consequently, the carbohydrate catabolism was different and the VFA profile changed significantly (Dijkstra 1994). This leads to the highest C2:C3 in *H. maculatum* and the lowest in *P. alpina*.

In vitro rumen methane production and kinetics

In terms of methane percentage, the two extreme values were recorded for *F. rubra* (28.9%), and *R. acris* (24.2%). The results obtained with *F. rubra* were mainly due to the chemical composition of this grass. The highest NDF content together with a low ADL value denote a rather high degradable fibre content that favoured the production of methane in the total gas. *R. acris* outcomes were more complex and unexpected because it presents a similar proximal composition to other plants. Moreover, the results obtained in the main fermentative parameters investigated do not differ markedly from those of other plants tested in the present study. The *Ranunculus* L. genus is known to have a rich profile of bioactive compounds with presumed therapeutic but also toxic effects (Reiné et al. 2020; Dai et al. 2024). However, to our knowledge, no study has yet found their possible implication in rumen fermentations. Considering total gas and methane (both in mL/g DMD), *P. erecta* was the plant that most limited their production despite the good DMD value. Nardin et al. (2023), in a study analysing the alkaloids of 62 pasture plants, saw that in *P.erecta* there is the presence of 8,10-Diethyllobelidiol hexoside a compound belongs to the piperidine group. Sousa et al. (2022) studied the effects of dietary supplementation with piperidine extract from *Prosopis juliflora* and found a reduction in energy loss such as methane in sheep. We can hypothesise that low production of methane could be related to the effect of 8–10 diethylbelidiol present in the *P. erecta* plant.

T. repens showed the greatest total VFA production (although not significant) and a high percentage of C2 compared to other plants and had a high methane concentration. It is known that VFAs, in particular C2, substantially determine the formation of H_2 which is converted by methanogenic Archaea into methane. In contrast to this, *P.alpina* showed the lowest C2 concentration, the lowest C2:C3 but a methane percentage similar to *T. repens*. Moreover, *H. maculatum* showed a comparable level of C2 to that of *T. repens* but a methane concentration is similar to the overall mean (25.8%). Macheboeuf et al. (2014) tested 156 plant species widespread in grassland and hedges with an *in vitro* system, finding a weaker correlation between methane and VFA production, suggesting that other aspects of fermentation influence methane production.

Protozoa were measured since Spanghero et al. (2022) demonstrated that their number is related to methane and ammonia emission in batch fermentation systems. However, the substrates used in the current study were unable to significantly influence overall protozoa counts, therefore methane and/or ammonia differences should be attributable to other factors.

Continuous methane monitoring during fermentation offers information on its kinetics, which supplements data from gas production and the VFA profiles of diverse substrates. In fact, substrates can reach similar methane yield throughout the fermentation but with different production kinetics (Braidot et al. 2023b). For example, two plants used in our experiment showed the same total methane yield but different patterns as can be observed in Figure 2. *P. alpina* had a rapid and intense methane production (short TMFR and high MFR), whereas *P. grandiflora* showed a different trend with a lower but longer methane production.

In the present experiment, *C. carvi*, *P. grandiflora*, and *R. acris* (Table 5) showed high values of MFR and the top values in the production rate decline ($A > 0.9$). However, these plants differed in the asymptotic methane percentage (B), with higher values reported for *P. grandiflora* when compared with the other two plants. This aspect is probably related to the different TMFR, *C. carvi* and *R. acris* are characterised by similar values while *P. grandiflora* was shown a smaller one. As a main consequence, *C. carvi* and *R. acris* quickly exhaust their maximum methane production, while *P. grandiflora* maintains the MFR longer (about half an hour), with a higher methane percentage.

The two grasses (*F. rubra* and *P. alpina*) together with *H. maculatum* have shown an opposite behaviour with lower values in MFR and in the decline of production rate ($A < 0.5$). This is partially compensated by a longer time to maximum fermentation rate (TMFR). Their kinetics parameters are probably due to their poor degradability and the high NDF content (Table

2), as noted in the PCA (Figure 1a). Despite the similarity between the grasses and *H. maculatum*, they had different asymptotic values for methane percentages. This aspect could be explained by considering the fibre content, in fact, *H. maculatum* showed the highest ADL level despite NDF content comparable with the two grasses.

A particular case is represented by *P. erecta*. Analysing its MFR and decay in production rate, the behaviour is similar to that observed for the two grasses and *H. maculatum*, but in contrast, it has a rather low TMFR value. However, it reaches an intermediate asymptotic value, demonstrating a gradual growth in the concentration of this gas over 24 h. In the literature, there are no studies on the effect of *P. erecta* on rumen fermentations, despite this species has several beneficial properties in human health, including anti-inflammatory and vasoconstrictor ones (Wölfle et al. 2017).

Conclusion

Numerous species compose alpine pastures and some contribute significantly to the composition of the diet of grazing cows. Among the plants we considered, their various chemical compositions influenced the fermentation parameters. Nevertheless, the percentage of methane produced, although varying significantly, always fell within a rather limited range (between 24% and 29%), despite relevant variations in the kinetics of production. Therefore, the study of methane kinetics adds further information, which allows us to identify plants with similar behaviours and to understand their possible effects on methane production over time. Further studies can make an important contribution to the topic by investigating other grazing environments in search of species that can decisively influence methane production.

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Ethical approval

Ethical review and approval were not required because this study did not involve animals for experimental or other scientific purposes.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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