ITALIAN JOURNAL OF ANIMAL SCIENCE
2025, VOL. 24, NO. 1, 711–727
https://doi.org/10.1080/1828051X.2025.2465696

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RESEARCH ARTICLE

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Alpine pasture herbs redirected hydrogen towards alternative sinks, inhibiting methane production: *in vitro* study

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ABSTRACT

The impact of six alpine herbs (*Alchemilla vulgaris* L., *Sanguisorba officinalis* L., *Tanacetum vulgare* L., *Cicerbita alpina* (L.) Wallr., *Galium odoratum* (L.) Scop., and *Sisymbrium officinale* (L.) Scop.), was compared with grass hay on rumen degradability and fermentation parameters. The seven plants were fermented using an automatic *in vitro* system to evaluate the kinetics of gas production (GP), degraded dry matter (dDM) and fermentation end products [volatile fatty acids (VFA), carbon dioxide, methane, and hydrogen]. Gas and methane productions were also computed from VFA using specific stoichiometric relationships. The partitioning factor (PF: ratio between dDM and GP) was calculated as an index of microbial growth. Compared to grass hay, the alpine herbs exhibited lower degradability (on average −12.8%) due to their high lignified fibre content. The alpine herbs also increase the PF (+9.1%), suggesting a reduction in microbial growth efficiency, and altered the VFA profile by increasing the proportion of acetic acid (+9.9%) at the expense of propionic (−11.9%) and n-butyric acids (−19.4%). Stoichiometric relationships typically associate these variations with an increase in methane proportion. However, this was not observed; in fact, *Sanguisorba officinalis* L. (−15.0%) and *Galium odoratum* L. Scop. (-13.9%) reduced methane production. The discrepancy between the measured and expected methane production indicates that part of the hydrogen, not used for methane synthesis, was redirected to alternative sinks such as reductive acetogenesis. This change in the fermentation profile appears to be modulated by bioactive compounds present in the medicinal herbs, which are potentially found in grazing pastures.

HIGHLIGHTS

- Bioactive compounds could be interesting for reducing methane emissions in cattle.
- At balsamic period, medicinal herbs exhibited pour degradability.
- Bioactive compounds affected rumen hydrogen utilisation, reducing methane production.

ARTICLE HISTORY
Received 1 August 2024
Revised 10 January 2025
Accepted 14 January 2025

KEYWORDS
Acetogenesis; alpine herbs; hydrogen recovery; methanogenesis; official plants

Introduction

The northern regions of Italy have an abundance of mountain meadows and grazing pastures, which are used during the summer transhumance periods (Bovolenta et al. 2008). These ecosystems have a rich diversity of fauna and, above all, flora, which includes a wide range of wild herbs that provide fodder to grazing animals along with other forages (Giupponi et al. 2006). Ruminants play a fundamental ecological role in biodiversity conservation (Sturaro et al. 2013) and in converting feed (fodder, pasture, and fibre-rich feedstuffs) to food with high nutritional value for humans, such as milk and meat. Thus, they ensure optimal and targeted use of impervious mountain lands and improve the attractiveness of mountain landscapes for tourism (Zendri et al. 2016). However, current research indicates that extensive grazing systems may lead to higher methane (CH_4) emissions per unit of production compared to intensive stall-based farming. This is primarily due to the lower productivity

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This article has been corrected with minor changes. These changes do not impact the academic content of the article.

levels of animals in extensive systems and their fibre-rich diets, which promote methanogenic fermentation processes (Bittante et al. 2018). During the summer transhumance period, ruminants graze on a variable quantity of spontaneous alpine herbs that grow in the ecosystem. These herbs are rich in secondary metabolites, such as tannins, polyphenols, essential oils, and saponins, which can modulate microorganism activity (Wencelová et al. 2014; Lima et al. 2019), the release of nutrients for the cow (e.g. volatile fatty acids, protein), and the emission of pollutants, like ammonia and CH_4. A few studies (Khiaosa-Ard et al. 2012; Macheboeuf et al. 2014; Cieslak et al. 2016; Boroja et al. 2018; Ineichen et al. 2019) have assessed the effect of these herbs on ruminal fermentation, diet degradability, and CH_4 emissions. Confirming these findings could highlight the environmental benefits of grazing-based farming while also supporting the optimisation of cow diets in intensive production systems.

The aim of this study was to compare *in vitro* rumen degradability, rumen microbial activity, end products of fermentation, and methanogenic potential of a common grass hay, *Lolium multiflorum* Lam., with six medicinal herbs (*Alchemilla vulgaris* L., *Sanguisorba officinalis* L., *Tanacetum vulgare* L., *Cicerbita alpina* (L.) Wallr., *Galium odoratum* (L.) Scop., and *Sisymbrium officinale* (L.) Scop.) typical of alpine pastures (in this specific case located in the Province of Trento, Italy).

Materials and methods

Ethics statements

All the experimental trials and procedures were carried out in accordance with Italian laws on animal welfare. All the methods used to obtain rumen fluid for the *in vitro* trials were approved by the Ethical Committee of the University of Padua, OPBA number 1312041/2022.

Herb preparation

The herbs tested were chosen from those that are edible for dairy cows and commonly found in the alpine pastures of Val di Non (Trento, Italy). They comprised the following 6 species: *Alchemilla vulgaris* L., *Sanguisorba officinalis* L., *Tanacetum vulgare* L., *Cicerbita alpina* (L.) Wallr., *Galium odoratum* (L.) Scop., and *Sisymbrium officinale* (L.) Scop. In addition, *Lolium multiflorum* Lam., harvested at the beginning of the earing stage, was used as the control (CTRL). The test herbs were harvested during their balsamic period and only the top parts of the shoots and leaves were retained and dried (Table 1). Freeze-drying was carried out at the Edmund Mach Foundation laboratories (San Michele all'Adige, Trento, Italy). About 200 g of each of the herb species were stored overnight at −80 °C before freeze-drying in a VirTis Benchtop K freeze dryer (SP Industries, USA). The freeze-drying process took place at −80 °C and 0.1-0.2 mmHg for 72 ± 1 h. The freeze-dried samples were then ground in an ultra-centrifugal mill (Retsch ZM 200, Retsch GmbH, Germany) with a grinding grid of 1 mm. The ground samples were used for both *in vitro* fermentation and chemical analysis.

Chemical composition of the herbs

The dry matter (DM) content of the herbs was determined by placing the samples overnight in an oven (Jointlab S.r.l., Italy) at 101–103 °C, in accordance with AOAC (2016) method 978.01. Ash content was determined by placing the samples in a muffle oven (Zetalab, Italy) at 550 °C for 4h, then weighing after cooling to ambient temperature in a desiccator, in accordance with AOAC (2016) method 942.05. Crude

Table 1. Herbs description.

Pasture herb[a]	Family[b]	Harvest stage[c]	Balsamic period[d]	Herb parts[e]
Lolium multiflorum Lam.[f]	Poaceae	Pre-blooming	–	Top and leaves
Alchemilla vulgaris L.	Rosaceae	Full bloom	May-August	Top and leaves
Sanguisorba officinalis L.	Rosaceae	Full bloom	April-August	Top and leaves
Tanacetum vulgare L.	Asteraceae	Full bloom	June-October	Top and leaves
Cicerbita alpina (L.) Wallr.	Asteraceae	Full bloom	May-July	Top and leaves
Galium odoratum (L.) Scop.	Rubicaceae	Full bloom	April-July	Top and leaves
Sisymbrium officinale (L.) Scop.	Brassicaceae	Full bloom	April-July	Top and leaves

[a]Pasture herbs: scientific name.
[b]Family: family affiliation.
[c]Harvest stage: stage of development at harvest.
[d]Balsamic period: maximum content of bioactive compounds.
[e]Herb parts: parts harvested.
[f]*Lolium multiflorum* Lam. was used in *in vitro* test as control.

Table 2. Chemical composition and total polyphenol content of herbs (g/kg DM) ($n = 2$).

Pasture herbs	DM, g/kg	CP	EE	aNDF	ADF	ADL	Ash	AIA	NSC	Total polyphenols content
Lolium multiflorum Lam.[a]	899.4	80.8	16.7	590.2	330.7	44.1	70.7	5.2	241.6	1.41
Alchemilla vulgaris L.	921.5	87.0	21.8	380.8	256.9	53.9	79.3	4.1	431.1	14.32
Sanguisorba officinalis L.	916.3	59.7	15.5	561.7	401.6	98.7	58.7	7.0	304.4	31.63
Tanacetum vulgare L.	933.4	97.5	14.9	606.5	443.6	108.4	79.7	4.6	201.4	6.59
Cicerbita alpina (L.) Wallr.	921.1	70.7	16.4	580.1	449.2	100.6	84.1	6.6	248.7	6.10
Galium odoratum (L.) Scop.	896.8	133.2	15.5	405.9	281.0	81.6	117.4	4.5	328.0	6.21
Sisymbrium officinale (L.) Scop.	941.4	81.3	8.2	697.4	508.8	92.2	57.5	4.3	155.6	0.93

DM: Dry Matter; CP: Crude Protein; EE: Ether Extract; aNDF: Neutral Detergent Fibre with heat stabile α-amylase and without sodium sulphite; ADF: Acid Detergent Fibre; ADL: Acid Detergent Lignin; AIA: Acid Insoluble Ash; NSC: Non-Structural Carbohydrates.
[a]*Lolium multiflorum* Lam. was used in *in vitro* test as control.

protein content (CP) was measured by the Kjeldahl method (AOAC, 2016, method 978.04) using a KjeltecTM 8400 analyser unit (Foss Electric A/S, Hillerød, Denmark). Ether extract (EE) was determined according to AOAC (2016) guidelines, method 2003.05. Fibre content was determined by the Van Soest et al. (1991) method. Neutral detergent fibre (aNDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) were determined with an Ankom fibre analyser 200 (Ankom, Rochester, NY, U.S.A.) instrument. The aNDF was determined with heat-stable α-amylase and without sulphite, in accordance with AOAC (2016) method 2002.04. Acid detergent fibre was determined following the AOAC (2016) method 973.18, after which the ADL content was determined sequentially with the same method. The chemical compositions of the herbs and the control plant are presented in Table 2.

Total polyphenol determination in herbs

The total polyphenol content of the herbs was analysed according to Di Stefano and Guidoni (1989) with some modifications. Briefly, to extract the polyphenols, 2.5 g of previously freeze-dried herbs were mixed with a 70:30 acetone:water solution. The mixture was homogenised, centrifuged at 10000 rpm for 10 min, and filtered to eliminate solid parts. A volume of 1–mL of extract was dried with a Rotavapor® R-300 (BUCHI s.r.l., Cornaredo, Milan, Italy), then resuspended with sulphuric acid (H_2SO_4) 1N in a 10–mL flask. The cartridge (Sep-Pak C18, 0.5 g; Waters) was activated with 2–mL of methanol and 5–mL of H_2SO_4 0.01N. One mL of the diluted sample was loaded onto the cartridge and washed with 2–mL of H_2SO_4 0.01N, then eluted in 2–mL of methanol and 5–mL of H_2O in a 20–mL flask. The extracted polyphenols were measured all together (total polyphenol content) by Folin–Ciocalteu reaction, a colorimetric method based on the chemical reduction of a reagent consisting of a mixture of molybdenum oxides and tungsten (Everette et al. 2010). This a standard procedure in wine analysis, but can also be applied to foodstuffs, such as fruits and vegetables. Total polyphenol content is reported as mg of catechin per kg of herb DM.

Experimental design and incubation procedure

The herbs were mixed with rumen fluid taken from 3 dairy cows and incubated in 3 consecutive *in vitro* runs conducted over consecutive weeks. Each run also included 3 blanks (fermentation batch without feed samples, one for each rumen fluid). In total, the experiment involved incubating a set of 72 artificial rumens: [(6 alpine herbs + 1 *Lolium multiflorum* Lam.)×3 rumen fluids × 3 *in vitro* runs + 9 blanks]. The rumen fluids were collected by oesophageal probe from fasting Simmental cows reared on the 'Lucio Toniolo' experimental farm of the University of Padua (Legnaro, Padua, Italy). The rumen fluid (1–L per cow) was maintained at 39 °C and promptly transported to the laboratory, where it was filtered through four layers of cheesecloth to remove coarse particles. All procedures were carried out under anaerobic conditions with a flow of CO_2. The buffer solution was prepared according to Menke and Steingass (1988), and kept at 39 °C with a flow of CO_2 for 45 min to maintain anaerobic conditions. Each fermentation bottle (322–mL total volume) was filled with 1.00 ± 0.01g of feed sample, 100–mL of buffer solution (Menke and Steingass 1988), and 50–mL of filtered rumen fluid. Finally, the bottle headspace (pre-heated to 39 °C) was filled with nitrogen (N_2) instead of CO_2 to maintain anaerobic conditions and avoid interference with total gas and CH_4 quantification (Park and Lee 2022). All the procedures from farm to incubation run were completed in under 40 min to ensure preservation of microflora activity.

The Ankom[RF] Gas Production System (Ankom Technology®, Macedon, NY, USA) was used to monitor gas production (GP) kinetics in all the fermentation bottles for 24h. Each bottle was equipped with a sensor designed to measure variations in pressure caused by microbial fermentation in the bottle headspace (172–mL). Each bottle was connected *via* a wireless antenna to a computer that continuously recorded the pressure values (RF GPM software version 11.4, Ankom Technology®, Macedon, NY, USA). After the incubation session, cumulative fermentation kinetics for each bottle were comprehensively evaluated through meticulous analysis of the curves generated by the GP software (Cattani et al. 2014).

Post-fermentation and sample collection

At the end of fermentation, the pH of each fermentation fluid was measured with a pH METRE BASIC20 (Crison Instruments, Barcellona, Spain). But, before opening the bottles, the gasses accumulated in the headspace of the bottles were collected with a 10–mL syringe and put into a 10–mL vacutainer. The samples were immediately analysed using the 490 Micro GC System (Agilent, California, USA) fitted with a thermal conductivity detector. The machine had two columns with different capillarity and work settings (one with argon as the carrier gas at 145 °C, 30 psi; the other with helium at 100 °C, 28 psi). The data collected from this procedure were analysed with the SOPRANE software (S.R.A. Instruments, France).

Two 4–mL aliquots were collected from each bottle and stored at −30 °C with 1–mL of metaphosphoric acid (25%, w/v) for ammonia N (N-NH_3) and volatile fatty acid (VFA) analyses. Ammonia N concentrations were determined with the ammonia rapid assay kit (K-AMIAR 02/20; Megazyme, Bray, Ireland). VFA concentrations were determined with a Jasco high-performance liquid chromatography instrument fitted with a PU-2080 pump (Tokyo, Japan), a model RI-2031 refraction index detector, a model AS-2055 autosampler, and a model CO-2060 column oven. Chromatographic separation was performed with an Aminex HPX 87H column (300 mm × 7.8 mm; Bio-Rad.), and the data interpreted with the ChromNAV software (Version 2.0, Jasco). Sulphuric acid 0.005 N was used as the mobile phase at a flow rate of 0.6 mL min^{-1}, elution gradient was isocratic, the volume of the sample injected was 20 µL (Falk et al. 2015). The calibration curves were obtained using the following volatile fatty acids standard: glacial acetic acid (Carlo Erba, CAS 64-19-7), propionic acid (Sigma-Aldrich, CAS 79-09-4), butyric acid (Fluka, CAS 107-92-6), iso-butyric acid (Sigma-Aldrich, CAS 79-31-2), valeric acid (Sigma-Aldrich, CAS 109-52-4), iso-valeric acid (Sigma-Aldrich, CAS 503-74-2), caproic acid (Sigma-Aldrich, CAS 142-62-1).

The microbial cellulolytic and proteolytic activity of rumen fluid during fermentation was assessed by the Fertimetro method (Nikolić et al. 2024). Three cotton threads (no.16) and three silk threads ('Royal Cocoon' no.24), 10 cm in length, from Cucirini Tre Stelle S.r.l. (Caleppio di Settala, Milan, Italy) were placed in each bottle. After incubation, the threads were retrieved, dried on absorbent paper, and immediately processed. The residual tensile breaking strength of the threads was measured with a digital dynamometer (IMADA ZP, ELIS Electronic Instruments and Systems, Rome, Italy), and microbial activity was quantified as a change in resistance relative to the non-incubated threads; the results were expressed in percentages.

Finally, the fluids in the bottles were filtered into glass crucibles por.3, and the residual dry matter (DMres) was extracted using a Fibertech FIWE Raw Fibre Extractor instrument (Velp Scientifica, Monza e Brianza, Italy).

Calculations

The non-structural carbohydrate (NSC) content was calculated for each herb using the following formula (Bovera et al. 2004):

$$\text{NSC (g/kg DM)} = 1000 - (\text{aNDF} + \text{EE} + \text{CP} + \text{Ash})$$

with all the constituents (aNDF, EE, CP, Ash) expressed as g/kg DM.

The *in vitro* degraded DM (dDM) was calculated as the difference between the initial amount of feed sample incubated (DM_{feed}) and the residual amount of DM (DM_{res}) after 24h of fermentation and corrected for the contribution of blanks (g); DM degradability was expressed per kg DM incubated (DMd, g/kg DM) (Goering and Van Soest, 1970).

$$\text{dDM (g)} = DM_{feed} - DM_{res}$$
$$\text{DMd (g/kg DM)} = (\text{dDM}/DM_{feed}) \times 1000$$

The cumulated gas production (GP) measured after 24h of incubation was corrected for the contribution of blanks and expressed per gram of DM incubated (mL/g DM) and per gram of dDM (mL/g dDM).

Microbial efficiency was estimated by calculating the partitioning factor (PF, mg/mL) as the ratio of dDM (mg) and cumulated GP (mL) after 24h of incubation (Blümmel et al. 1997).

Metabolic hydrogen recovery ($[2H]_{recovery}$) was calculated as described by Marty and Demeyer (1973) as the ratio between the hydrogen accepted and the hydrogen released from the substrate fermentation. The following equation was based on the mMol of VFA produced (corrected for blanks contribution) and the actual mMol of CH_4 and H_2 produced after 24h of fermentation:

$$[2H]_{recovery}(\%) = \frac{(2 \times Propinate) + (2 \times Butyrate) + (4 \times CH_4) + H_2}{(2 \times Acetate) + Propionate + (4 \times Butyrate)} \times 100$$

The gases composition (H_2, and CH_4) measured after 24h was corrected for the contribution of blanks and expressed as volume proportions (% v/v), amounts produced per gram of DM incubated (mL/g DM), and per gram of dDM (mL/g dDM).

Ammonia N (N-NH_3) was expressed as mMol/L, and the individual VFAs were expressed as proportions of total VFA (g/100g VFA). The ratio of acetic acid plus n-butyric acid to propionic acid was calculated [(A + B)/P, w/w].

Finally, gas (GP_{VFA}) and CH_4 production (CH_{4VFA}) were also computed from the VFA profile (corrected for blanks contribution) using the stoichiometric equations proposed by Blümmel et al. (1999), and were expressed as volume proportions (% v/v), per gram of DM incubated (mL/g DM), and per gram of dDM (mL/g dDM).

Statistical analysis

The R software v.4.1.1 (R Core Team 2021) was used for the statistical analysis with the following mixed model:

$$y_{ijkl} = \mu + Herb_i + Rumen\ fluid_j + Run_k + e_{ijkl}$$

where y_{ijkl} is the observed trait; μ is the overall mean; $Herb_i$ is the fixed effect of the *i*th herb ($i = 6$ alpine herbs+*Lolium multiflorum* Lam. as control); $Rumen\ fluid_j$ is the random effect of the *j*th donor cow ($j = 3$ rumen fluids); Run_k is the random effect of the *k*th *in vitro* incubation run ($k = 3$ *in vitro* runs); and e_{ijkl} is the residual random error term. All random effects and the residuals were assumed to have a normal distribution with a mean of zero and a variance σ^2_n.

Orthogonal contrasts ($p < 0.05$) were built for all traits studied, and compared the effect of each herb with the effect of *Lolium multiflorum* Lam. In addition, some traits were compared with each other in order to obtain the R^2 (determination coefficient) and the linear regression equation. The relationships with an R^2 greater than 0.50 are graphically represented in Figures 3 to 8.

Results

Alpine herbs: composition and total polyphenol content

The chemical compositions and total polyphenol contents of the six tested herbs and *Lolium multiflorum* Lam. (CTRL) are reported in Table 2. In general, almost all the herbs and CTRL had a low CP content (less than 10% DM), except *Galium odoratum*, which had a CP content of more than 13% DM. The fibre content of the alpine herbs was highly variable, whether aNDF, ranging from 38% to 70% of DM, or ADF, ranging from 25% to 50%. *Alchemilla vulgaris* was the only herb with a lignin content similar to the CTRL (ADL 4–5% DM), while all the others were highly lignified (ADL 8–11% DM). Generally, the total polyphenol content, expressed as g of catechin per kg of herb DM, ranged between 0.93 and 31.63 g/kg DM. *Sisymbrium officinale* had the lowest content, and *Sanguisorba officinalis* the highest, followed by *Alchemilla vulgaris* (14.32 g/kg DM).

Pasture herbs degradability and products of fermentation

The least square means (LSM) and the effects of the herbs tested on the fermentation parameters *in vitro* are presented in Table 3. pH, DMd (g/100 g DM), GP (mL/g DM), PF (mg/mL), and the degree of cotton degradation differed significantly between herbs, while GP (mL/g dDM), N-NH_3 (mMol/L) and the degree of silk degradation did not ($p > 0.05$). *Alchemilla vulgaris* and *Sanguisorba officinalis* had lower DMd than the CTRL, while *Galium odoratum* had higher degradability. Similarly, *Alchemilla vulgaris* and *Sanguisorba officinalis*, as well as *Tanacetum vulgare* had a lower GP (mL/g DM) compared with CTRL ($p < 0.001$). *Tanacetum vulgare*, *Cicerbita alpina*, and *Galium odoratum* had a higher PF than the CTRL ($p < 0.05$). Furthermore, the degree of cotton thread degradation was significantly lower in *Alchemilla vulgaris* and *Sanguisorba officinalis* than in the CTRL ($p < 0.001$) but remained unaffected when incubated with the other herbs.

Table 4 shows the LSMs of the VFA profiles after 24h of incubation, along with the orthogonal contrasts for each herb compared with *Lolium multiflorum* Lam. Overall, the VFA profiles of the tested herbs significantly differed from the CTRL, with iso-butyric acid being the only one VFA unaffected by the herbs. Generally, compared with CTRL, the herbs increased the relative amounts of acetic acid at the expense of propionic acid and n-butyric acid ($p < 0.01$). However, *Cicerbita alpina* and *Sisymbrium officinale* did not affect the proportion of propionic acid, and *Alchemilla vulgaris* did not reduce the proportion of n-butyric acid.

The various herbs had different effects on the proportions and amounts of H_2 and CH_4 (Table 5). *Alchemilla vulgaris*, *Sanguisorba officinalis*, and *Tanacetum vulgare* lowered the production of both H_2 and CH_4 (mL/g DM) compared with the CTRL.

Table 3. *In vitro* rumen fermentation traits (after 24h) of pasture herbs compared to *Lolium multiflorum* Lam. ($n = 9$).

Pasture herbs	pH	DMd, g/100g DM	GP, mL/g DM	GP, mL/g dDM	PF, mg/mL	N-NH$_3$, mMol/L	Cotton Fertimetro, % of degradation	Silk Fertimetro, % of degradation
Lolium multiflorum Lam.[a]	6.75	42.70	165.0	412.0	2.60	10.13	86.60	10.19
Alchemilla vulgaris L.	6.89***	31.10**	124.0***	469.0	2.73	9.89	54.70***	11.29
Sanguisorba officinalis L.	6.97***	24.20***	108.0***	516.0	2.38	9.95	39.30***	11.41
Tanacetum vulgare L.	6.93***	36.40	123.0***	349.0	3.10*	9.84	84.80	11.47
Cicerbita alpina (L.) Wallr.	6.85***	42.80	148.0	344.0	3.02*	9.61	82.50	12.27
Galium odoratum (L.) Scop.	6.79	50.70*	167.0	332.0	3.16*	9.42	85.10	9.67
Sisymbrium officinale (L.) Scop.	6.84**	38.10	150.0	396.0	2.63	9.51	81.50	14.28
SE	0.02	2.70	10.25	64.30	0.29	0.35	7.01	3.49
P-value	***	***	***	ns	*	ns	***	ns

[a]*Lolium multiflorum* Lam. was used in *in vitro* test as control.
DMd: Dry Matter degradability; GP: Gas Production; N-NH$_3$: ammonia nitrogen; SE: Standard Error.
ns: non-significant; * *P*-value < 0.05; ** *P*-value < 0.01; *** *P*-value < 0.001.
The least square means with superscript asterisks are significantly different from the *Lolium multiflorum* Lam. according with the results of the contrasts.
The *P*-value in the last row refers to the significance of the treatments in the statistical model.

Table 4. Volatile fatty acid proportions (g/100g VFA) after 24h of incubation of pasture herbs compared to *Lolium multiflorum* Lam. ($n = 9$).

Pasture herbs	Acetic acid	Propionic acid	Iso-butyric acid	N-butyric acid	Iso-valeric acid	N-valeric acid	Caproic acid	Branched acids[a]	(A + B)/P ratio[b]
Lolium multiflorum Lam.[c]	52.70	19.90	2.20	19.50	2.65	1.73	1.36	6.59	3.64
Alchemilla vulgaris L.	56.30**	15.80***	1.96	18.60	3.22*	2.36***	1.83*	7.51	4.74***
Sanguisorba officinalis L.	57.30***	16.80***	2.28	17.70*	2.93	1.41	1.70	6.59	4.47***
Tanacetum vulgare L.	59.30***	16.90***	2.65	13.00***	3.68***	2.64***	1.91**	8.93***	4.29***
Cicerbita alpina (L.) Wallr.	58.40***	19.60	2.50	14.20***	2.72	1.49	1.18	6.68	3.71
Galium odoratum (L.) Scop.	59.50***	16.90***	2.41	15.70***	2.75	1.37*	1.37	6.50	4.47***
Sisymbrium officinale (L.) Scop.	56.70***	19.20	2.53	15.10***	3.09	1.88	1.52	7.48	3.75
SE	0.86	0.37	0.44	0.57	0.31	0.15	0.22	0.51	0.09
P-value	***	***	ns	***	**	***	***	**	***

[a]Branched acids: iso-butyric acid + iso-valeric acid + n-valeric acid.
[b](A + B)/P ratio: (acetic acid + n-butyric acid)/propionic acid.
[c]*Lolium multiflorum* Lam. was used in *in vitro* test as control.
ns: non-significant; * *P*-value < 0.05; ** *P*-value < 0.01; *** *P*-value < 0.001.
The least square means with superscript asterisks are significantly different from the *Lolium multiflorum* Lam. according with the results of the contrasts.
The P-value in the last row refers to the significance of the treatment in the statistical model.

Table 5. *In vitro* gas composition and production (hydrogen, H_2; methane, CH_4) after 24h of incubation of pasture herbs compared to *Lolium multiflorum* Lam. ($n = 9$) and efficiency.

Pasture herbs	CH_4, mL/g DM	H_2, mL/g DM	CH_4, % v/v	H_2, % v/v	H_2, mL/g dDM	CH_4, mL/g dDM	H_2 recovery index, %
Lolium multiflorum Lam.[a]	29.20	0.86	18.00	0.54	2.11	71.20	93.1
Alchemilla vulgaris L.	20.20**	0.56**	16.00	0.46	2.03	81.50	76.6**
Sanguisorba officinalis L.	16.60***	0.47***	15.30*	0.44	2.22	86.50	79.5*
Tanacetum vulgare L.	21.00**	0.64*	17.20	0.52	1.77	61.70	70.0***
Cicerbita alpina (L.) Wallr.	25.60	0.71	16.90	0.48	1.68	61.20	70.8***
Galium odoratum (L.) Scop.	25.90	1.27***	15.50*	0.80**	2.49	51.90	70.2***
Sisymbrium officinale (L.) Scop.	27.40	0.86	18.50	0.58	2.26	74.00	83.7
SE[2]	2.39	0.08	0.80	0.06	0.26	15.10	6.22
P-value	***	***	**	***	ns	ns	**

[a]*Lolium multiflorum* Lam. was used in *in vitro* test as control.
ns: non-significant; * *P*-value < 0.05; ** *P*-value < 0.01; *** *P*-value < 0.001.
The least square means with superscript asterisks are significantly different from the *Lolium multiflorum* Lam. according with the results of the contrasts.
The P-value in the last row refers to the significance of the treatment in the statistical model.

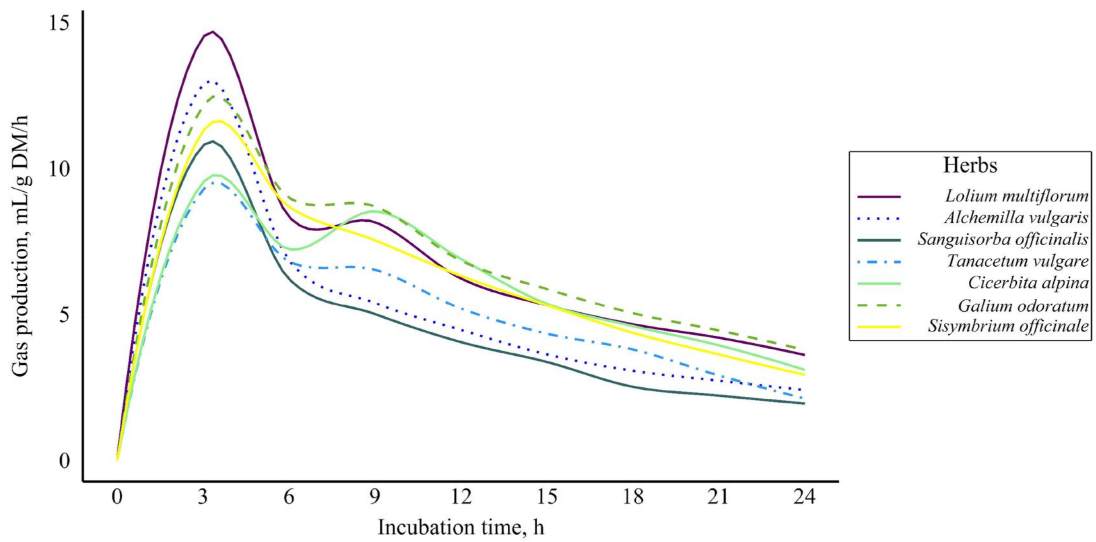
Sanguisorba officinalis also changed the composition of the fermentation gases by slightly reducing the proportion of CH_4 ($p < 0.05$, % v/v). On the other hand, *Galium odoratum* reduced the proportion of CH_4 ($p < 0.05$, % v/v) in the fermentation gases without significantly reducing the amount of CH_4 produced (mL/g DM), and at the same time it increased both the proportion ($p < 0.01$) and amount ($p < 0.001$) of H_2. When the volume data were expressed as mL/g dDM, there were no differences between the herbs.

Regarding the predictions of GP and CH_4 production according to the stoichiometric equation

Table 6. *In vitro* gas production (GP_{VFA}) and CH_4 production (CH_{4VFA}) computed from VFA production after 24h of incubation of pasture herb compared to *Lolium multiflorum* Lam. (Blümmel et al. 1999) ($n = 9$).

Pasture herbs	GP_{VFA}, mL/g DM	GP_{VFA}, mL/g dDM	CH_{4VFA}, % v/v	CH_{4VFA}, mL/g DM	CH_{4VFA}, mL/g dDM
Lolium multiflorum Lam.[a]	163.0	414.0	16.30	26.70	67.50
Alchemilla vulgaris L.	124.0*	479.0	18.80***	23.20	89.10
Sanguisorba officinalis L.	102.0***	504.0	18.40***	18.70**	92.60
Tanacetum vulgare L.	149.0	419.0	18.90***	27.90	79.30
Cicerbita alpina (L.) Wallr.	171.0	398.0	17.60***	30.00	70.10
Galium odoratum (L.) Scop.	185.0	370.0	18.80***	34.60**	69.60
Sisymbrium officinale (L.) Scop.	165.0	434.0	17.30**	28.70	74.90
SE^2	13.60	74.50	0.22	2.38	13.50
P-value	***	ns	***	***	ns

[a]*Lolium multiflorum* Lam. was used in *in vitro* test as control.
ns: non-significant; * *P*-value < 0.05; ** *P*-value < 0.01; *** *P*-value < 0.001.
The least square means with superscript asterisks are significantly different from the *Lolium multiflorum* Lam. according with the results of the contrasts.
The P-value in the last row refers to the significance of the treatment in the statistical model.



	Incubation time, h							
Herbs	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**
Alchemilla vulgaris			***	***	***	***	***	***
Sanguisorba officinalis	***	**	***	***	***	***	***	***
Tanacetum vulgare	***	*	**	*	**	**	***	***
Cicerbita alpina	***							
Gallium odoratum	**				*			
Sisymbrium officinale	***						*	*

Figure 1. *In vitro* gas production per h of incubation (mL/g DM/h) of pasture herbs. In the table below are reported the contrasts among each herb and the *lolium multiflorum* lam. (CTRL). The significances by 3–hours interval of incubation are reported.
Lolium multiflorum Lam. was used in *in vitro* test as control.
The asterisks reported in the figure represent the level of significance for the difference between the single herb and the *Lolium multiflorum* Lam., according with the results of the contrasts (absence: non-significant difference; * *P*-value < 0.05; ** *P*-value < 0.01; *** *P*-value < 0.001).

proposed by Blümmel et al. (1999), there were few differences between the herbs and CTRL (Table 6). Only two herbs, *Alchemilla vulgaris* and *Sanguisorba officinalis*, decreased GP_{VFA} (mL/g DM) compared with the CTRL, while all the tested herbs greatly altered the estimated gas composition by increasing CH_4 proportion ($p < 0.001$; CH_{4VFA}, % v/v). However, when CH_4 production was expressed as a quantity (mL/g DM), it was reduced only by *Sanguisorba officinalis* compared to CTRL and was increased by *Galium odoratum*.

Rates and cumulative kinetics of gas production

Figure 1 depicts the *in vitro* kinetics of the GP rate (mL/g DM/h) for each herb. All the kinetics exhibited a biphasic pattern, with a first peak at 3h and a second peak at between 9 and 12h, depending on the herb incubated. The GP rate of the herbs was lower ($p < 0.001$) than that of the CTRL, either for the entire duration of the incubation (*Sanguisorba officinalis* and *Tanacetum vulgare*), within the first 3h of incubation (*Cicerbita alpina, Galium odoratum* and *Sisymbrium* officinale), or after 9h of fermentation (*Alchemilla vulgaris*). Figure 2 depicts the kinetics of cumulative gas production (GP) over 24 h of incubation (mL/g DM). The curves show that the pasture herbs have a lower GP than the control (CTRL), with notable differences in the shape of the kinetics among the various herbs.

Linear regressions

Figure 3 highlights a strong positive correlation (R^2: 0.87) between NSC (g/kg DM) and DMd (g/100g DM) in five of the seven herbs tested. *Alchemilla vulgaris* and *Sanguisorba officinalis* were excluded from the regression because of a high NSC content and a low DMd (square symbol in the figure).

The positive correlation between DMd and cumulative GP is evident in Figure 4, whether measured at 24h of incubation (GP, mL/g DM; R^2: 0.84; Figure 4(a)) or computed from the VFA profile (GP_{VFA}, mL/g DM; R^2: 0.94; Figure 4(b)). Furthermore, DMd positively correlated with the PF (mg DMd/mL GP; R^2: 0.63; Figure 4(c)) and with the degree of cotton degradation (% degradation; R^2: 0.74; Figure 4(d)), which are indicators of the efficiency of converting feed into microbial mass (as summarised in Scheme 1) and an index of microbial activity, respectively.

Linear regression among the VFAs produced revealed a negative correlation only between acetic acid and n-butyric acid (Figure 5), but no significant correlations among the other fatty acids.

The linear regression between the measured and the computed GP (GP and GP_{VFA}, mL/g DM) is shown in Figure 6, and in this case, the two parameters were highly correlated (R^2: 0.82) and the trend was positive.

Figure 7 depicts the linear regression between the measured and computed CH_4. When expressed as mL/g DM, the trend was positive, and the R^2 approached the threshold of 0.50 (Figure 7(a)). However, when the data were expressed as proportion of the fermentation gases (% v/v), the trend was negative, indicating a decrease in CH_4 production as the computed CH_4 increased (Figure 7(b)). In both cases, the values for *Alchemilla vulgaris* and *Sanguisorba officinalis* were extreme compared with the other herbs. Figure 8 depicts the linear regression between the ratio of (acetic + n-butyric acids)/propionic acid and CH_4 production, both measured (% v/v; 8 A) and computed (% v/v; 8B). Figure 8(a) shows a decreasing linear pattern with an R^2 of 0.65, while Figure 8(b) shows an increasing linear pattern with an R^2 of 0.77.

Discussion

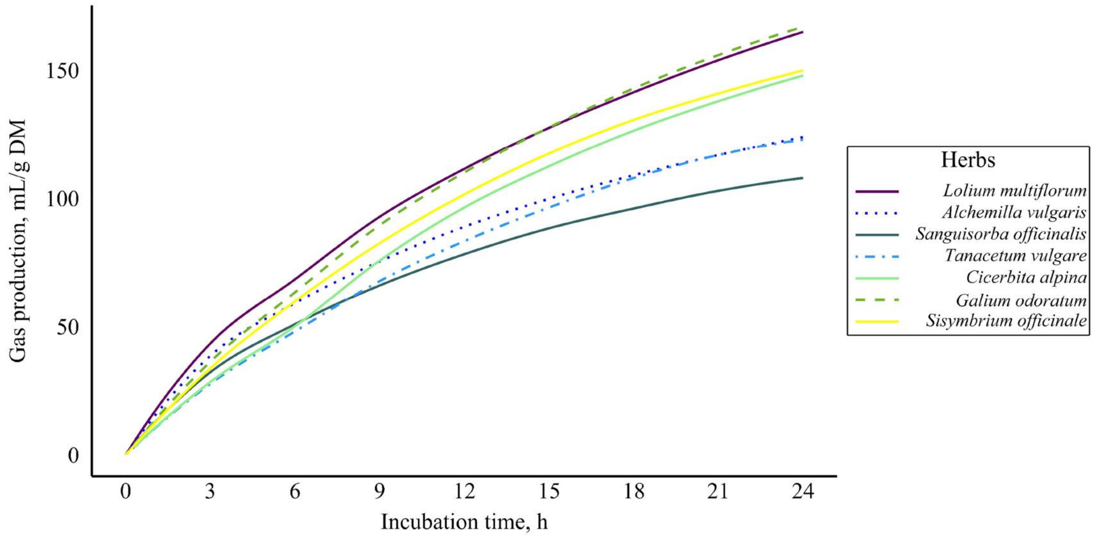
Pasture herb composition and balsamic time

The chemical composition of forages depends on various aspects such as the botanical characteristics of herbs, the environmental and seasonal conditions, agronomic techniques, and, above all, the maturation stage of the herbs at harvest (Nelson and Moser 1994; Palumbo et al. 2021). The pasture herbs characterised in this study were harvested during their balsamic period, when the bioactive substances in their tissues are at maximum concentrations (Basso et al. 1998). There is very little information regarding the palatability of alpine herbs, nor the cows' feeding behaviours and their actual intake at grazing (Palumbo et al. 2021). Usually, the balsamic time of officinal plants coincides with blooming, hence at an advanced stage of plant maturation. This may partly account for the chemical composition as well as the low degradability of the herbs compared with CTRL. As shown in Table 1, all the herbs investigated have different balsamic periods, all coinciding with full blooming, which depends on the family of affiliation.

Degradability of pasture herbs

The degradability of fodder is mainly related to the contents of easily fermentable carbohydrates or fibre, tissue lignification, and the content of inhibitory substances, such as polyphenols (Bovolenta et al. 2008; Bizzuti et al. 2023). As depicted in Figure 3, the degradability of herbs primarily depends on the NSC content, although this is not the case for *Alchemilla vulgaris* and *Sanguisorba officinalis*, which exhibited the lowest degradability despite having the highest NSC content (Tables 2 and 3). This is probably because these two herbs were rich in polyphenols, which seem to strongly inhibit fermentation processes. These results, therefore, provide confirmation that the rumen utilisation of forages depends on a combination of factors, including interactions between the different chemical constituents of the plant (Li 2021).

Several studies have shown that the most prevalent category of polyphenols in *Alchemilla vulgaris* and *Sanguisorba officinalis* are condensed tannins (Cieslak et al. 2016; Boroja et al. 2018; Jurić et al. 2020). These affect rumen microbial activity as well as diet and protein degradation (Wencelová et al. 2014; Bueno et al. 2015; Aboagye and Beauchemin 2019). Cieslak et al. (2016) showed that extract of *Sanguisorba officinalis* exerts an inhibitory effect on feed digestibility, which



	Incubation time, h							
Herbs	3	6	9	12	15	18	21	24
Alchemilla vulgaris		*	**	**	***	***	***	***
Sanguisorba officinalis	***	***	***	***	***	***	***	***
Tanacetum vulgare	***	***	***	***	***	***	***	***
Cicerbita alpina	***	***	**	*				
Gallium odoratum	**							
Sisymbrium officinale	***							

Figure 2. *In vitro* cumulated gas production (mL/g DM) of pasture herbs measured for 24h. In the table below are reported the contrasts among each herb and the *lolium multiflorum* lam. (CTRL). The significances are reported by 3–hours interval of incubation.
Lolium multiflorum Lam. was used in *in vitro* test as control.
The asterisks reported in the figure represent the level of significance for the difference between the single herb and the *Lolium multiflorum* Lam., according with the results of the contrasts (absence: non-significant difference; * *P*-value < 0.05; ** *P*-value < 0.01; *** *P*-value < 0.001).

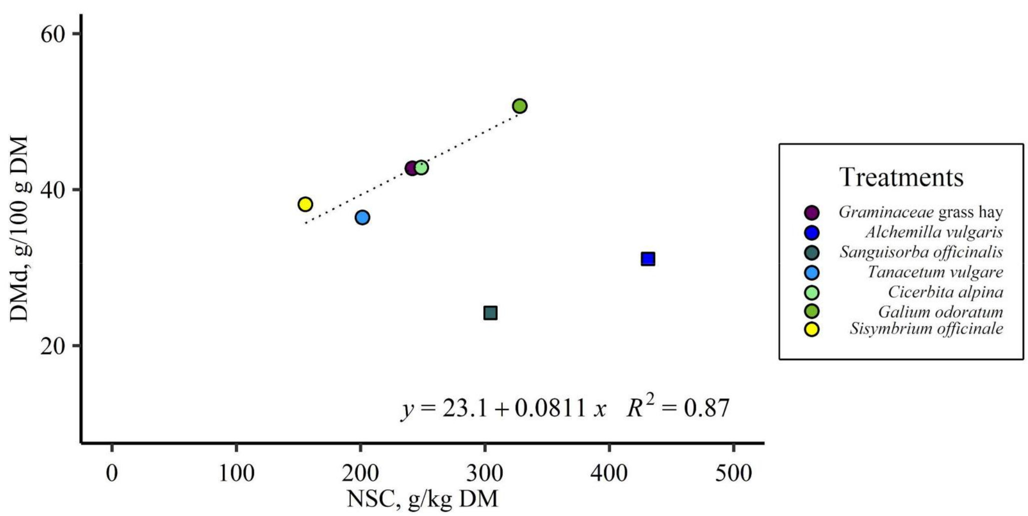


Figure 3. Linear regression between non-structural carbohydrates (NSC, g/kg DM) and dry matter degradability (DMd, g/100g DM).
Alchemilla vulgaris and *Sanguisorba officinalis* were excluded by the regression because of their differences respect to the other herbs. The two herbs are represented as square symbol (□) in the figure.

is linked to a reduction in cellulolytic bacteria activity in the rumen.

Irrespective of the chemical characteristics of the herbs, fermentation substrate disappearance was positively correlated with GP (Figure 4(a)) and with GP_{VFA} (Figure 4(b)). Substrate disappearance also positively correlated with the PF (Figure 4(c)), suggesting that increasing feed degradability facilitates conversion

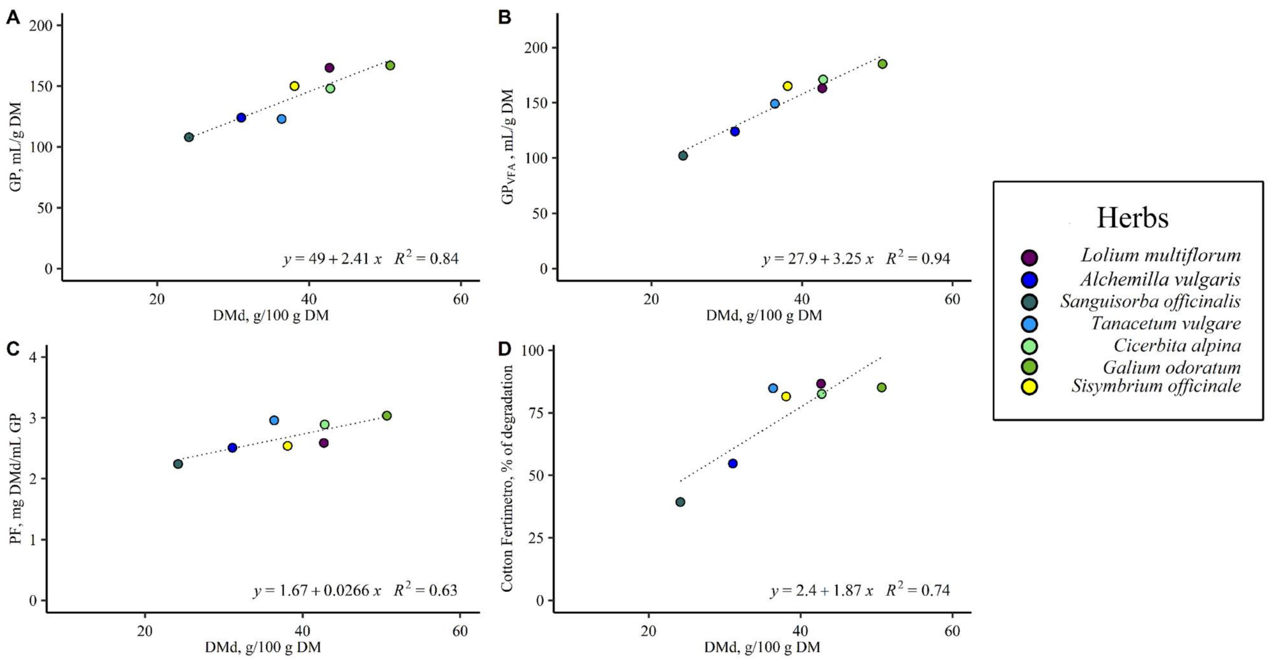
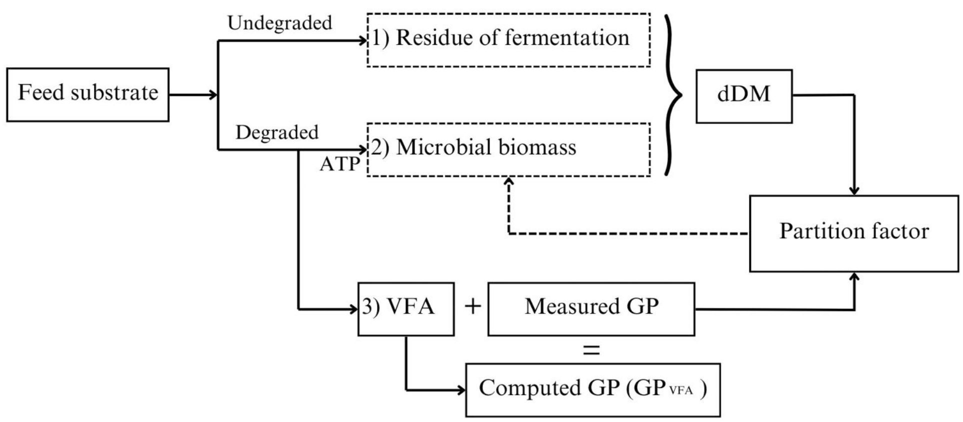


Figure 4. Linear regression between the dry matter degradability (DMd, g/100g DM) and the following *in vitro* parameters: (a) gas production measured at 24h of incubation (GP, mL/g DM), (b) gas production computed from VFA (GP_{VFA}, mL/g DM), (c) partitioning factor (PF, mg dDM/mL GP); (d) microbial activity expressed as fertimetro degradation (% of cotton thread degradation).



Scheme 1. Representation of feed carbon repartition during the fermentation process, 1) undegraded residues, 2) microbial mass, and 3) fermentation byproducts (volatile fatty acids, VFA; gas production, GP). Due to the stoichiometric equation, computed GP was calculated (GP_{VFA}). Partitioning factor is an indicator of the substrate conversion efficiency into microbial mass (ratio between degraded dry matter (dDM, g) and GP (mL).

of the substrate into microbial mass rather than into VFA and gases, as recently reported by Bizzuti et al. (2023) for a variety of feed by-products (Scheme 1). Bueno et al. (2020) observed that when extract of condensed tannins was added to cows' diets, degradability, VFA, and GP decreased, but the PF increased. This discrepancy in the results regarding the efficiency of microbial utilisation of feeds may have to do with whether the tannins are integrated into the feed structure or added as an extract. When tannins encrust the cell wall, they can inhibit microbial attack and fibre degradation because they form stable complexes that are difficult for microorganisms to degrade. Consequently, microbial enzymatic activity is reduced, limiting the ability of the microorganisms to break down plant fibre. Tannins added as extracts may also exert a binding effect on soluble compounds (dietary and bacterial proteins), reducing protein turnover and improving fermentative efficiency. The DMd of herbs also correlated with the degree of cotton thread degradation using the Fertimetro method (Figure 4(d)), but the two parameters appear to offer different types of information. As previously discussed, degradability depends on a complex interaction between the

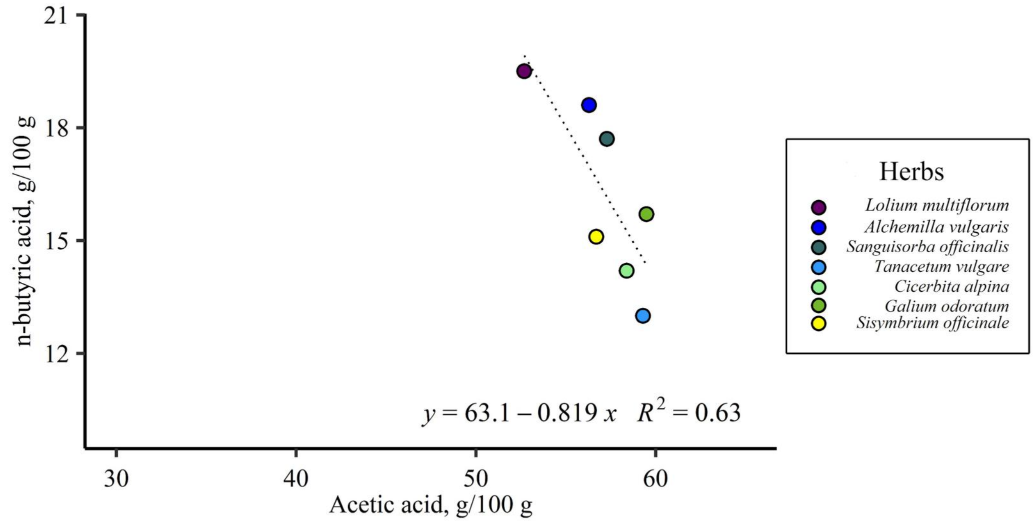


Figure 5. Linear regression between the proportion of acetic acid (g/100g) and the proportion of n-butyric acid (g/100g).

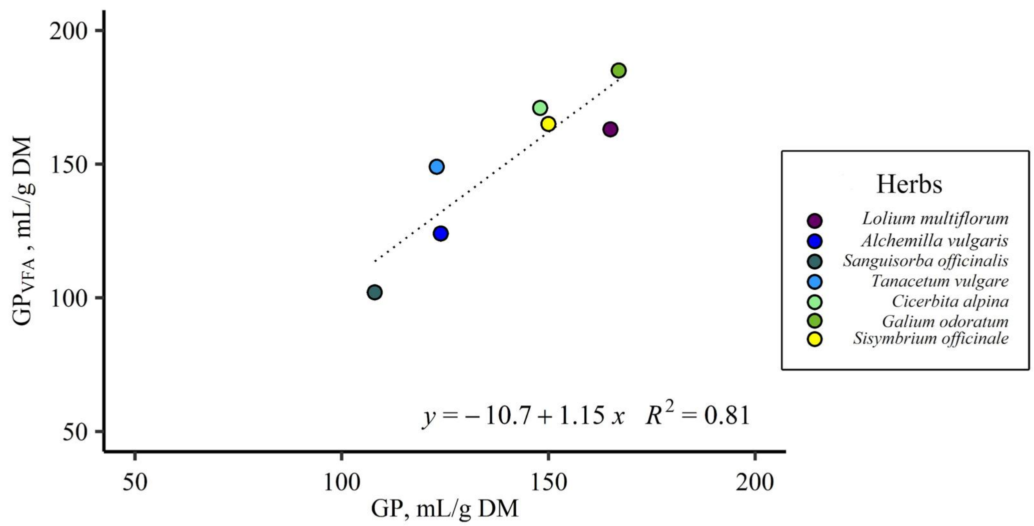


Figure 6. Linear regression between gas production measured (GP, mL/g DM) and computed from VFA production (GP_{VFA}, mL/g DM).

chemical constituents and bioactive compounds of the feed. Cotton degradation, on the other hand, represents the thread's loss of tensile strength due to microbial fermentation and therefore acts as a proxy for microbial activity in rumen fluid (Nikolić et al. 2024). In the present study, the thread degradation rates of *Alchemilla vulgaris* and *Sanguisorba officinalis* appeared to provide a measure of the inhibitory effects of tannins on rumen microbial activity.

Gas production kinetics of pasture herbs

The pasture herbs tested exhibited differences in fermentation kinetics (Figure 1). Compared with CTRL, only *Sanguisorba officinalis* and *Tanacetum vulgare* reduced fermentability throughout the entire 24h incubation period. Moreover, the trend in the fermentative processes indicates that the fermentability of all the herbs, with the exception of *Alchemilla vulgaris*, was low compared with the CTRL in the first 3h of incubation. Fermentation kinetics describes the rate at which rumen microflora use dietary components, and, in relation to the rumen flow rate, determines the effective degradability of dietary components in the rumen. This is why low feed fermentability during the first hours has a large impact on nutrient production in the rumen, because it can increase the escape of undegraded particles from the rumen (Millen et al. 2016). Similarly, low degradability in the first few hours extend the time the feed remains in the rumen (Rodrigues et al. 2002). However, it is also true that the cumulative GP at 24h is also related to GP

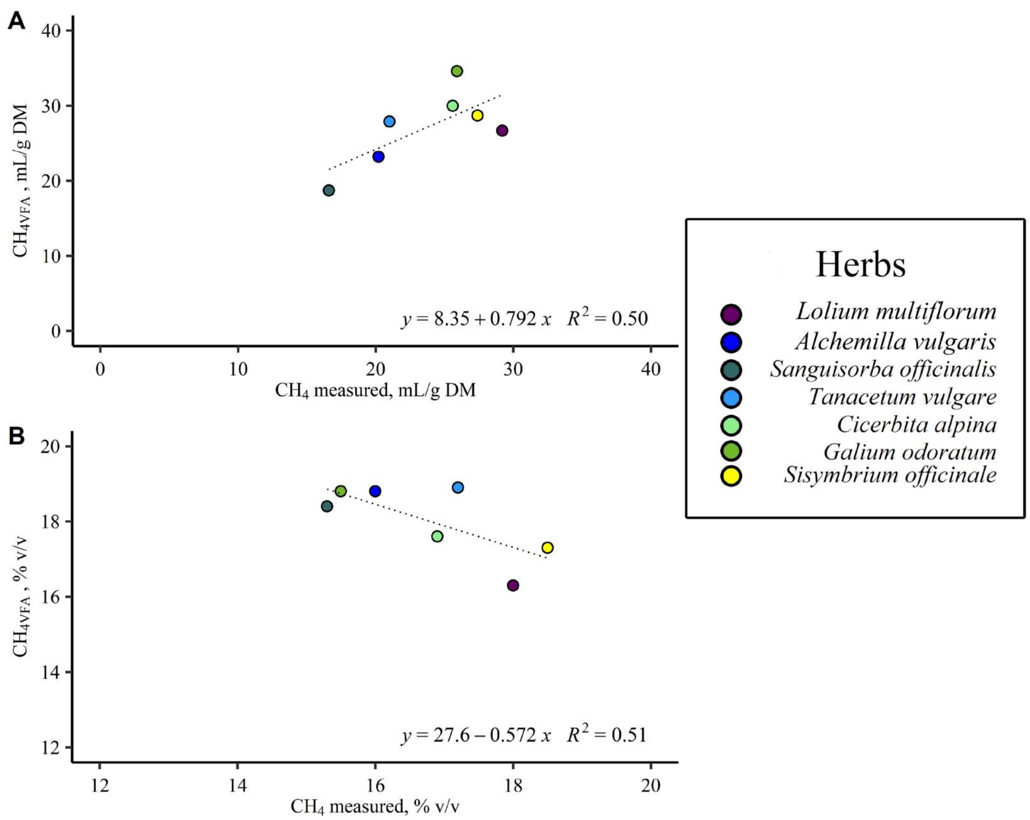


Figure 7. Linear regression between CH_4 measured and computed from VFA (CH_{4VFA}), both expressed as (a) amounts (mL/g DM) and (b) proportions (% v/v).

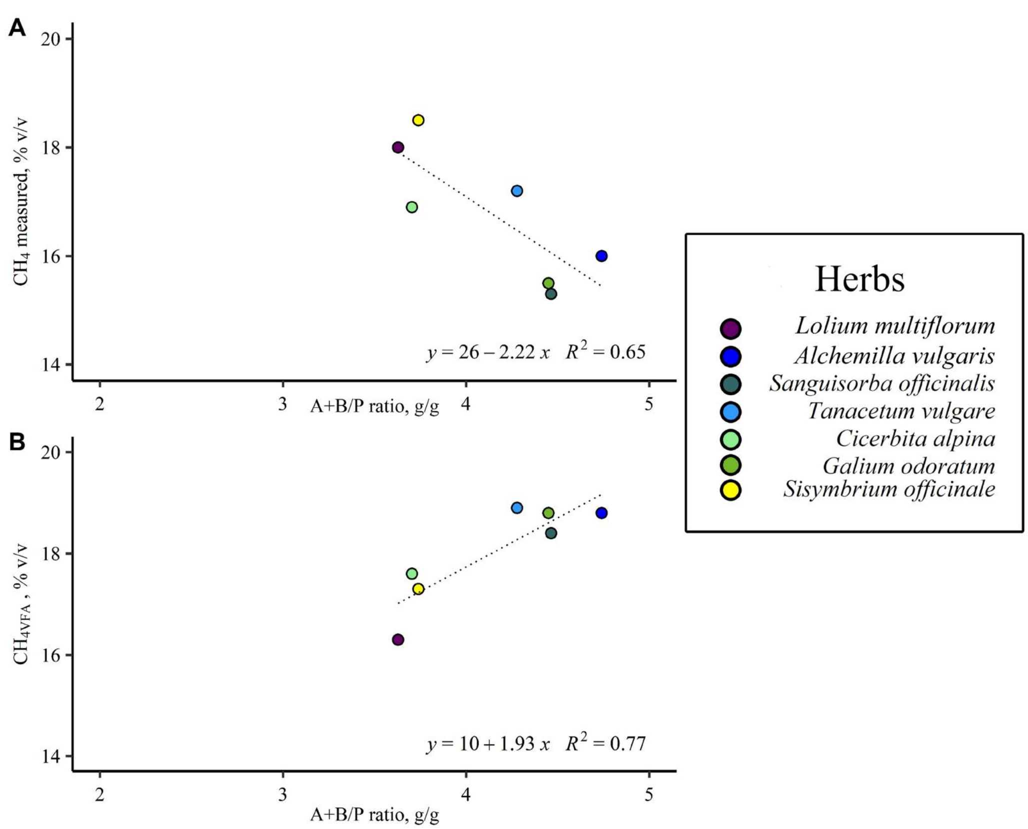


Figure 8. Linear regression between the ratio of (acetic + n-butyric)/propionic acid (a + B/P ratio, g/g) and CH_4 proportion (a) measured (CH_4, % v/v) and (b) computed from VFA (CH_{4VFA}, % v/v).

measured at an early stage (Figure 2), and GP at 24h (as well as degradability at 48h) was historically chosen to predict the energy value of feeds given the high repeatability of these measurements compared with those obtained at early incubation times (NRC, 2001).

The GP rate (Figure 1) for some herbs (i.e. *Tanacetum vulgare*, *Cicerbita alpina*, and *Galium odoratum*) and for CTRL exhibited a biphasic pattern with two distinct peaks of fermentation, one after 3h, the other after 9h of incubation. This pattern may be linked to the degradation rates of the various chemical constituents of the herbs and the time required by ruminal microflora to break down NSC compared with slowly degradable fibrous fractions.

Products of the fermentation of pasture herbs: volatile fatty acid profiles and gas composition

All the pasture herbs tested were associated with an increase in acetic acid production with corresponding reductions in the proportions of propionic and n-butyric acids (Table 4). This pattern is in line with previous findings that forage-rich, high-fibre diets favoured acetic acid production at the expense of propionic acid (Beckett et al. 2021). Bioactive compounds, on the other hand, appear to exert contradictory effects. Patra (2010) found that both tannins and essential oils resulted in a quadratic decrease in the acetate/propionate ratio with inhibition of CH_4 production. In contrast, Kholif and Olafadehan (2021) observed an increase in acetic acid production accompanied by a decrease in propionic acid with the use of tannins in general, as well as specific condensed tannins and a few essential oils (e.g. peppermint, cinnamaldehyde).

Fibre fermentation typically results in high acetic acid production associated with an increase in n-butyric acid, whereas the fermentation of starch and soluble carbohydrates in the rumen is associated with higher levels of propionic acid (Sutton et al. 2003). However, ruminal microorganisms are also responsible for high VFA interconversion during fermentation, involving an interchange between acetic acid and n-butyric acid, especially with high-fibre diets (Sutton et al. 2003). This exchange process may explain the observed inverse relationship between the proportions of acetic acid and n-butyric acid following the fermentation of alpine herbs, as depicted in Figure 3. However, the conditions that potentially favour this process of interchange have not yet been fully elucidated. Hess et al. (2006) noted a linear decrease in the acetic acid to n-butyric acid ratio in the rumen when legumes rich in tannins were added to diets.

VFA production is closely correlated with the production and composition of fermentation gases, mainly CO_2 and CH_4, according to precise stoichiometric relationships (Blümmel et al. 1999). Figure s 6 and 7 present the linear regressions between cumulative GP and CH_4 production and calculated using the stoichiometric relationships derived from VFA production. As expected, GP and GP_{VFA} were closely correlated. However, the regression between CH_4 and CH_{4VFA} was significantly weaker, and when the data were expressed as a proportion (% v/v), they exhibited an unexpected weak negative correlation. This result is in line with observations made by Macheboeuf et al. (2014), who carried out *in vitro* screening of 156 herbs and found a strong correlation between measured and predicted gas, whereas the correlation appeared to be much weaker for CH_4. Macheboeuf et al. (2014) suggest that the fermentation of certain feed produces substances in different quantities to those predicted by stoichiometric equations. This indicates that, while stoichiometric equations can accurately predict overall GP, they are not as reliable for predicting CH_4 (Figure 8). This could be due to several factors, including the presence of bioactive compounds in pasture herbs that can influence microbial fermentative activity, the metabolic pathways of carbohydrate utilisation, and the repartition of H_2 among different sinks, such as fermentation products and microbial biomass.

In this study, we primarily focused on ruminal energy metabolism, with only limited indicators available for protein metabolism. However, the modest effects of medicinal plants and their bioactive compounds on ammonia and branched-chain fatty acid concentrations, primarily derived from amino acid fermentation, suggest that these compounds did not significantly influence protein catabolism or turnover. Conversely, several *in vitro* studies have reported reductions in NH_3 and branched-chain VFAs when various types of tannins were added to the feed substrate (Bhatta et al. 2009; Battelli et al. 2023). Therefore, these findings do not support the hypothesised protein-binding effect of bioactive compounds on dietary and microbial proteins, nor the potential positive impact on microbial biomass increase.

However, the activity of these compounds can explain the differences between CTRL and pasture herbs regarding both measured (Table 5) and predicted CH_4 values (Table 6). In quantitative terms (mL/g DM), pasture herbs produced less fermentation gas, including CH_4, compared to CTRL. As previously discussed, these quantitative differences are mainly due to the low degradability of alpine herbs. In terms of the gas profile (% v/v), alpine herbs did not increase the CH_4, as would be expected from CH_{4VFA}. In the case of *Sanguisorba officinalis* and *Galium odoratum*, the CH_4 proportions were lower than those of the CTRL (% v/v). Only *Galium odoratum* exhibited a reduction in CH_4 proportion, which is in line with the increase of H_2 proportion. The H_2 increase during incubation may be associated with inhibition of the activity and growth of microorganisms linked to the methanogenesis process (Choudhury et al. 2022). On this issue, Cieslak et al. (2016) and Aboagye and Beauchemin (2019) reported that secondary bioactive compounds could cause an accumulation of gaseous H_2 *in vitro* by inhibiting the utilisation of hydrogen in CH_4 synthesis. However, in stoichiometric terms, the increase in gaseous H_2 production observed with *Galium odoratum* accounts for only a small proportion (6.8%) of the reduced hydrogen utilisation for CH_4 production. Therefore, given that dissolved H_2 does not accumulate in the rumen fluid, it is evident that for all pasture herbs the lower-than-expected CH_4 production implies the presence of metabolic pathways that incorporate hydrogen into alternative sinks (Aboagye and Beauchemin 2019). The measure of this alternative sink is indicated by the $H_{2recovery}$ index, which was as much as 90% for CTRL (Marty and Demeyer 1973), but was significantly lower for all the pasture herbs. The reduction in the $H_{2recovery}$ index could, as reviewed by Beauchemin et al. (2020), be explained by reductive acetogenesis, a metabolic pathway through which acetate is produced from hydrogenation of carbon dioxide ($2CO_2 + 2H_2 \rightleftarrows CH_3COOH + 2H_2O$) (Demeyer 1991). This process could explain the reduction in fermentation GP (CO_2 and CH_4) and the increase in the acetic acid concentration relative to propionic and n-butyric acids. Reductive acetogenesis is a desirable process of hydrogen incorporation because acetate is a source of energy. From a stoichiometric perspective, the acetogenesis metabolic pathway can largely explain the reduced utilisation of hydrogen resulting from methanogenesis inhibition and the consequent low $H_{2recovery}$ index observed with alpine herbs. Assuming that the greater production of acetic acid obtained with alpine herbs compared with CTRL is linked to acetogenesis, this process could account for 140% of the hydrogen derived from the inhibition of methanogenesis, although with relevant variability among the herbs tested (ranging from 98% for *Cicerbita alpina* to 204% for *Alchemilla vulgaris*). However, attempts to induce acetogenesis in the rumen *in vivo* have been unsuccessful (Fievez et al. 1999) because reductive acetogenesis is thermodynamically outcompeted by methanogenesis in the normal rumen ($\Delta G = -131$ kJ *vs* -95 kJ) (Ungerfeld and Kohn 2006). It appears that the ruminal environment contains a small microbial population of acetogenic bacteria and that *in vitro* fermentation processes may not accurately reflect *in vivo* ruminal conditions (Joblin 1999). Nevertheless, it is a potentially beneficial hydrogen sink that could be enhanced in methanogenesis-inhibited rumen fermentation. In theory, diverting hydrogen away from methanogenesis towards fermentation end-products that the host animal can absorb and utilise, as well as towards microbial biomass synthesis may reduce CH_4 emissions and potentially improve the host animal's productivity. However, this potential has not been consistently realised so far.

Conclusions

In the present work, we evaluated the effect of six alpine herbs on ruminal fermentation characteristics, gas production, and CH_4 production *in vitro* using *Lolium multiflorum* Lam. as control. The alpine herbs, harvested during their balsamic period, demonstrated low gas production, primarily due to a poor rumen degradability, alongside a decrease in microbial growth efficiency. However, this was partially offset by a reduction in CH_4 production compared to expectations based on the VFA profile. This suggests that some of the hydrogen not used for CH_4 synthesis and microbial growth was redirected towards an alternative sink, such as acetogenesis. This shift in the fermentation profile seems to be influenced by the bioactive compounds present in alpine herbs and available in grazing pastures. However, further research is needed to better understand the action of these types of herbs on rumen pathways, and specifically how bioactive compounds could modulate the production of CH_4 by dairy cows.

Disclosure statement

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

Funding

This study was funded within the Interconnected Nord-Est Innovation Ecosystem (iNEST) and received funding from the European Union Next-GenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) – MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.5 – D.D. 1058 23/06/2022, ECS00000043). This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them. This work was also supported by the BIRD213117/21 (by the University of Padua) and is part of the FoodTraNet project that has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No. 956265.

Data availably statement

The data presented in this study are available on request from the corresponding author upon reasonable request.

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