Livestock Science 309 (2026) 105968

Contents lists available at ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

The methanogenic potential of grass-fed dairy calves is mostly impacted by the feeding system, not the genotype

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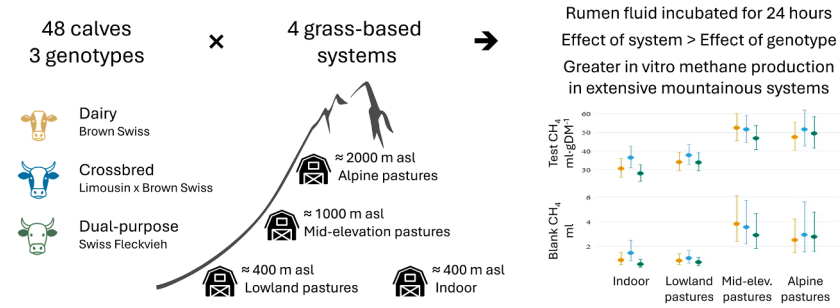
HIGHLIGHTS

- We raised 48 calves from three genotypes in one indoor and three pasture systems.
- Each calf's rumen fluid was analysed in vitro for gas, methane and ammonia production.
- Methane yield was greater in montane than in lowland and indoor systems.
- Genotype effects were significant but smaller than system effects.
- Time between rumen sampling and lab analysis was accounted for in models.

GRAPHICAL ABSTRACT



Mesbahi et al. The methanogenic potential of grass-fed dairy calves is mostly impacted by the feeding system, not the genotype

ARTICLE INFO

Keywords:
Greenhouse gas
Hohenheim gas test
Male calf
Multispecies sward
Ruminal fermentation

ABSTRACT

Grass-based veal production offers a sustainable and welfare-oriented use for male dairy calves. Multispecies pastures are often linked to lower methane emissions, yet this relationship has not been investigated in veal calves. This study evaluated the in vitro gas production of rumen fluid from six-month-old male calves reared for three months under four grass-based systems: indoor hay feeding, and grazing on lowland, mid-elevation, or alpine pastures. Three genotypes were compared – Brown Swiss (dairy), Limousin × Brown Swiss (crossbred), and Swiss Fleckvieh (dual-purpose) – with four calves of each genotype per system (n = 48). All calves received hay ad libitum and standardised feed supplements. Access to pasture totalled 611, 769, and 771 h for the lowland, mid-elevation, and alpine pasture systems, respectively. Rumen fluid was collected by intubation (day 175 ± 9) and incubated in vitro to assess total gas, methane (CH_4), and ammonia (NH_3) production from a standard feed, and in vitro organic matter digestibility (IVOMD) was calculated. Absolute CH_4 production in samples from extensive systems (mid-elevation and alpine pastures) was up to 49 % higher than in intensive systems (indoor and lowland pastures). IVOMD was greatest in indoor samples, while NH_3 formation significantly differed between systems. Rumen fluid from Swiss Fleckvieh calves yielded less CH_4 than that of crossbred calves, with no genotype × system interactions. Overall, rumen fluid from more intensive, less botanically diverse systems produced lower in vitro gas emissions. The methodological influence of time between sampling and incubation was quantified and discussed.

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https://doi.org/10.1016/j.livsci.2026.105968
Received 12 November 2025; Received in revised form 22 April 2026; Accepted 4 May 2026
Available online 6 May 2026

1. Introduction

Plant-based mitigation of methanogenesis in ruminants is a large field of research, often focused on individual plants and their phytochemical composition and effects (Arndt et al., 2022). From a practical standpoint, the challenge of scaling up the production of effective plant-based methane mitigators is often overlooked, yet it poses a major challenge due to the large land areas required, land that could otherwise be used for food crops (Dittmann and Leiber, 2024). In contrast, multispecies swards may offer a more land-efficient solution, as they can be cultivated on the same fields already used for forage production, or integrated into crop rotations within mixed farming systems (Cooledge et al., 2022; Dumont et al., 2022). Therefore, the methane-inhibiting potentials of multispecies forages are the subject of current research (Carmona-Flores et al., 2020; Hassan et al., 2025; Loza et al., 2021), although results have been contrasting. The effects could be linked to plant secondary metabolites, particularly phenols like tannins, which are known to modulate the rumen microbiota (Niderkorn and Baumont, 2009).

Another ruminal process with important environmental implications is the fermentative degradation of protein. It affects feed nitrogen use efficiency and determines the amount of nitrogen excreted, which in turn influences soluble nitrogen losses to water and gaseous emissions (Epper et al., 2025). At the rumen level, elevated ammonia concentrations indicate inefficient microbial protein synthesis or oversupply of rumen-degradable protein, leading to nitrogen losses from feed (Reynolds and Kristensen, 2008). Phytochemicals such as tannins can also affect these processes; thus, similar to their role in methane mitigation, certain herbs may improve nitrogen use efficiency in ruminants while reducing urinary losses of soluble nitrogen compounds (Kapp-Bitter et al., 2023; Macheboeuf et al., 2014).

However, effects of forages on rumen processes, such as methanogenesis and ammonia formation, are not solely driven by plant secondary compounds. They also depend on nutrient balances, which are influenced by the plant's nutrient composition, growth stage (da Cunha et al., 2023), and the intensity of forage production.

While much of the in vivo research on methanogenesis has focused on dairy cows, beef production faces similar challenges (Smith et al., 2022). This also applies to young animals such as calves raised for fattening or replacement heifers, who are often overlooked regarding their contribution to overall emissions (Müller-Kiedrowski, 2025; Staerfl et al., 2012).

In organic dairy production, a specific challenge concerns male calves from dairy herds, which are early sold into conventional markets due to high costs and low profitability of raising them (Rell et al., 2022). One promising alternative under evaluation in Switzerland is to rear these calves on extensive, species-rich pastures, which may offer ecological and economic benefits while improving the welfare of the fattening calves (Pfeifer et al., 2025; Spengler Neff et al., 2023). Mountain pastures are often botanically diverse and rich in plant secondary compounds, such as tannins and phenols (Jayanegara et al., 2011; Willems et al., 2014) which can influence rumen fermentation. In such systems, both the quality of the pasture and the genotype of the calf, whether pure dairy or crossbred with a beef sire, are important factors influencing feed conversion efficiency. Moreover, when considering environmental impacts such as methanogenesis and ammonia formation, both diet composition (Jayanegara et al., 2011; Müller-Kiedrowski, 2025) and genotype (Scholtz et al., 2023) play a significant role.

However, measuring emissions in grazing animals remains a relevant challenge. Respiratory chambers are often considered a gold standard, also for calves (Cristobal-Carballo et al., 2021), but their use requires bringing animals from the pasture to a research centre, which may impact the animals' feed intake and bias the measurements (Goopy et al., 2016). Also, the logistical demands of measurements in respiration chambers limit the sample size. The sulphur hexafluoride (SF6) tracer gas technique is used in adult sheep (Ulyatt et al., 2005) and cattle (Orcasberro et al., 2021), but its application in young calves remains limited, partly due to practical constraints related to animal size and equipment. In addition, the logistical constraints of repeated gas sampling across multiple and remote pasture sites limit its feasibility in the present study. Mobile methane detectors like the GreenFeed system (Meale et al., 2021) are expensive if applied to a large number of animals at different sites and are challenging to transport across remote alpine pastures.

In vitro techniques are commonly used to evaluate various forages in terms of methane and ammonia formation, as well as organic matter digestibility (Kapp-Bitter et al., 2021a; Menke and Steingass, 1988; Soliva et al., 2008). In addition to comparing feeds, in vitro tests can also be used to compare rumen fluids collected from animals under different feeding regimes (Khiaosa-ard et al., 2012). To the knowledge of the authors, this approach has not previously been applied on a large scale where individual animals, rather than feeds, serve as the experimental unit. This novel design presents specific methodological challenges, particularly regarding sample transport and the time lag between collection and incubation, which are discussed in the present study.

Against the described backgrounds and methodological challenges, the current study investigates the effects of pasture type and calf genotype on methanogenesis and ammonia formation potentials, using a novel adaptation of the Hohenheim in vitro batch test (Menke and Steingass, 1988) that allows standardized yet animal-specific assessment of rumen fermentation. We hypothesize a relationship between pasture botanical composition and methanogenesis, mediated by plant secondary compounds such as phenols and tannins.

2. Material and methods

The animal experimentation procedure was reviewed and approved by the ethics committee of the canton of Argovia, Switzerland (application number 36489 79700).

2.1. Study design

The study was conducted on 48 calves from May to September 2024. The experimental design followed a fully crossed factorial structure, with calves distributed across three genotypes and four farming systems. The genotypes included Brown Swiss (BS, dairy), Limousin × Brown Swiss (L × BS, crossbreed), and Swiss Fleckvieh (SF, dairy-oriented dual purpose). The farming systems corresponded to four sites located in Switzerland: a hay-based indoor system and three pasture-based systems representing lowland, mid-elevation, and alpine pastures (Fig. 1).

Calves were purchased at an age of 34 d (± 9) from different farms, aiming for as low variance as possible in calving dates. During a pre-experimental phase (until 3 months of age), all calves were housed indoors on straw bedding and received the same feed ration, composed of powdered milk (51 kg·calf^{-1}), maize pellets (19 kg DM·calf^{-1}), and alfalfa pellets (5.5 kg DM·calf^{-1}), which were individually allocated. Hay was offered ad libitum, and intake was estimated at the group level (average: 45 kg DM·calf^{-1}). This phase aimed to control for the effects of early nutrition before the experimental period and took place in two sites: one for the indoor and lowland pasture systems, and another for the mid-elevation and alpine pasture systems. At the end of the third month of life, calves were divided into four groups, balanced as evenly as possible for age (95 ± 9 days) and body weight (125.1 ± 11.2 kg), while ensuring that any pairs born on the same farm were assigned to different groups. Respectively, four calves of each genotype were allocated to each system.

During the experimental phase (95 to 175 ± 9 days of age), calves were assigned to either an indoor hay-based system or one of three pasture-based systems. The same quantity of powdered milk was provided across all systems until calves reached 4 months of age (Table 1). Maize pellets, alfalfa pellets, soy-free concentrate, and grass silage were

individually distributed twice daily in the barn. Feeding levels differed between systems, with higher amounts provided in the indoor system than in the pasture-based systems, and grass silage provided only indoors (Table 1). Hay was available ad libitum in all systems' barns, and water was available ad libitum both in the barns and at pastures. Calves in the pasture-based systems had access to pasture for approximately 10 h per day and remained in the barn for the remaining time. Grazing occurred either during the day or at night depending on temperatures, while total grazing duration varied between systems, depending on weather and soil conditions, resulting in total grazing durations of 611, 769, and 771 h for the lowland, mid-elevation, and alpine systems, respectively. Feeding in the barn was conducted individually, whereas pasture intake was not individually controlled.

Pastures of the lowland system (around 400 m above sea level) were composed of 26 species, dominated by *Lolium perenne* (27 %), *Trifolium repens* (20 %), and *Agrostis stolonifera* (9 %). Pastures from the mid-elevation system (around 1000 m asl) regrouped 32 species, dominated by L. *perenne* (24 %), *T. repens* (19 %), and *Poa pratensis* (17 %). Pastures from the alpine system (around 2000 m asl) were composed of 61 species, dominated by *Carex* sp (27 %), *Alchemilla vulgaris* (18 %), and *Deschampsia flexuosa* (8 %).

During the week preceding rumen fluid sampling (day of life 175 ± 9), the pastures of the lowland, mid-elevation and alpine systems were respectively composed of 73, 50 and 66 % of grasses, 22, 26 and 4 % of legumes, and 5, 24 and 30 % of forbs (detailed quantities and nutritive values of pastures and feeds during this last week are summarized in Supplementary Table 1).

2.2. Feed phenols and tannins contents

Feeds were collected in the week preceding the rumen fluid sampling to analyse their contents in total extractable phenols (TEP), non-tannin phenols (NTP), and condensed tannins (CT), as described in Kapp-Bitter et al. (2023). Briefly, to measure the TEP and NTP contents, the feed material was extracted in acetone, incubated with polyvinylpolypyrrolidone, and centrifuged. TEP (from acetone extract) and NTP (from polyvinylpolypyrrolidone extract) were measured at 725 nm on a spectrophotometer (Bio Spectrometer Eppendorf D30) after reaction with Folin–Ciocalteu solution. To measure CT content, the acetone extract was incubated with ammonium iron (III) sulphate and butanol-HCL, and measured at 550 nm on a spectrophotometer (Bio Spectrometer Eppendorf D30). TEP and NTP contents are calculated against the gallic acid standard, while CT content is calculated as leucocyanidin equivalents.

Table 1
Description of the feeding rations.

Feed	Indoor hay-based system	Pasture-based systems
Powder milk	7 kg DM·calf^{-1}	7 kg DM·calf^{-1}
Hay	Ad libitum	Ad libitum in barns (~14 h·day^{-1})
Maize pellets	144 kg DM·calf^{-1}	93 kg DM·calf^{-1}
Alfalfa pellets	68 kg DM·calf^{-1}	40 kg DM·calf^{-1}
Soy-free concentrate	48 kg DM·calf^{-1}	15 kg DM·calf^{-1}
Grass silage	92 kg DM·calf^{-1}	None
Pasture access	None	~10 h·day^{-1}

2.3. Rumen fluid sampling

Calves were sampled for rumen fluid at 175 ± 9 days of age (*x00A0;*> 0.05 between systems). Body weight at sampling averaged 187 ± 26 kg and differed between systems ($P < 0.05$), with mean values of 220 ± 15, 188 ± 15, 166 ± 23, and 174 ± 11 kg for the indoor, lowland, mid-elevation, and alpine systems, respectively. The sampling took place on the farms via oesophageal tubing, between 0900 h and 1230 h, using a Ruminator-like manual vacuum pump connected to a flexible tube and a perforated sampling probe, following the recommendations from the SmartCow consortium (Muizelaar et al., 2020). Briefly, 200 ml of rumen fluid was sampled after discarding at least the first 50 ml that contained saliva. The rumen fluids were filtered through a cheese cloth into insulated bottles pre-warmed at 39°C, from which the air had been expelled. The bottles were kept warm from the farm to the lab. Each site was sampled on a different day to ensure that rumen fluid collection was performed by the same operators, thereby minimizing potential operator-related bias. Samples were transported as soon as possible from the farm to the lab, thus the time lag between sampling and in vitro fermentation is only due to the transportation time and differs between systems. On all sites except the mid-elevation grassland system, calves were fasted for three hours before we started the rumen fluid sampling, i.e. from 0600 h. Calves from the mid-elevation grassland system were kept on pasture until the rumen fluid sampling by mistake.

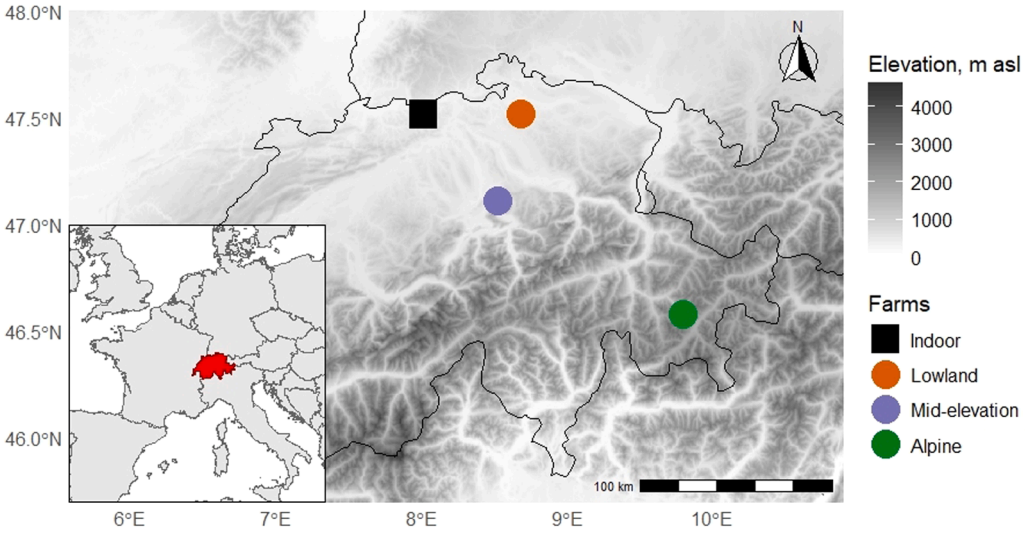

Fig. 1. Location of the four experimental sites. The sites include an indoor hay-based system (400 m above see level) and three pasture-based systems: lowland (400 m asl), mid-elevation (1000 m asl), and alpine (2000 m asl).

2.4. In vitro fermentation experiment

The in vitro experiment followed the protocol of the Hohenheim Gas Test (Menke and Steingass, 1988). In vitro incubations were conducted separately for each farming system, with each system processed on a different day as soon as possible after rumen fluid collection. Within each system, all samples were incubated in a single run. For each calf, test and blank fermenters were set up and started simultaneously. The pH of the rumen fluid was first measured using a pH meter (Seven Excellence pH meter S400 equipped with InLab Science Pro-ISM pH electrode, Mettler Toledo, Switzerland). For each calf, an individual fermentation medium was prepared by mixing rumen fluid with buffer solution. Four fermenters were prepared per calf: two received 30 ml of the medium only and served as blanks, while the other two were pre-loaded with 200 mg of standard hay provided by Hohenheim University before receiving 30 ml of the same medium, serving as test samples. Every fermenter was immediately placed in an incubator with a rotating disc and maintained at 39°C for exactly 24 h.

After 24 h, the fermentation gas volume was recorded using the calibrated scale printed on the fermenters. Gas samples (250 μl) were analysed for CH_4 concentrations by gas chromatography using an Agilent 8860 GC System equipped with a modified S/SI EPC Luer Lock injection system (Teckso GmbH, Germany) and a Micropacked ShinCarbon ST 80/100 column (2 m, 0.53 mm ID; Restek, Germany). The carrier gas was argon (Ar 5.0) with a column flow rate of 3.4 ml·min^{-1}. The oven was held isothermally at 80 °C. The injection was performed with a 1:1 split ratio, and the thermal conductivity detector was used with a total run time of 8.3 min. Data were analysed using OpenLab CDS software (Agilent Technologies). The pH of the medium was measured again, and the medium from the test fermenters was stored at -20°C for subsequent NH_3 analysis using a Gas Sensitive Electrode GSE (Seven Excellence pH meter S400 equipped with a GSE Ammonia Electrode, Type 15 230 3000, and a temperature probe ATC NTC 30k Ohm, Mettler Toledo, Switzerland). All fermenters (i.e. blank and test) were analysed. An estimate for in vitro organic matter digestibility (IVOMD) was then calculated according to the equation from Menke and Steingass (1988), by: $IVOMD\ (mg \cdot g) = 148.8 + 8.893 \times [GP\ test\ (ml \cdot 200\ mg\ DM) - GP\ blank\ (ml)] + 0.448 \times CP\ (mg \cdot g) + 0.651 \times ash\ (mg \cdot g)$ where GP test = gas production from the test fermenter after 24 h of incubation, GP blank = gas production from the blank fermenter after 24 h of incubation, and CP = crude protein content of the standard hay.

2.5. Statistical analysis of data

The Hohenheim gas test is typically used to compare fodders. In that context, gas production from blank fermenters – which contain only incubation medium and no fodder – is subtracted from gas production in test fermenters to isolate the effect of the fodder itself. However, since the present study aimed to compare emissions at the animal level – not just from the fodder – we chose not to subtract blank values. Instead, we analysed test fermenters and blank fermenters separately. Gas production from test fermenters reflected the potential emissions from calves digesting a standardized fodder, while gas production from blank fermenters represented the basal fermentation activity of the calves' rumen fluid alone.

Statistical analysis was conducted using R v 4.3.1 (R Core Team, 2023). For each fermentation characteristic – including total gas production, methane production and concentrations, ammonia production, IVOMD, and rumen fluid pH – we fitted a generalized linear model using 'glmmTMB' v 1.1.10 (Brooks et al., 2017). The model included the effects of farming system, genotype, their interaction, and the time elapsed between sampling and analysis. The time between sampling and analysis was included as a covariate to control for potential degradation or bias due to sample storage time.

To test for the overall effects of predictors, we used the 'emmeans' package v 1.10.4 (Lenth, 2021), which provides an ANOVA-like table based on Wald χ^2 statistics. Estimated marginal means were computed, and pairwise comparisons were performed to assess significant differences between farming systems and genotypes. All multiple comparisons were adjusted using the Tukey method using 'multcomp' v 1.4–26 (Hothorn et al., 2002). Model fit was assessed using Nagelkerke's coefficient of determination (pseudo-R^2) and root mean square error (RMSE), calculated with 'performance' v 0.12.3 (Lüdecke et al., 2021).

In order to allow a fully replicable analysis, the dataset and the script are freely accessible online (Mesbahi et al., 2026).

3. Results

3.1. Feeds phenols and condensed tannins content

The contents of TEP, NTP and CT in hay and pellets were similar between farming systems (averaging 7.7, 6.8 and 0.4 g·kg^{-1} for hay, and 5.7, 5.2 and 4.2 g·kg^{-1} for pellets). However, pasture TEP, NTP and CT contents differed between systems (Fig. 2, Supplementary Table 1). Compared to lowland pasture, TEP content was 46 % greater in mid-elevation pasture and 162 % greater in alpine pasture; NTP content increased by 42 % in mid-elevation pasture and 25 % in alpine pasture; and CT content was 4 % greater in mid-elevation pasture and 504 % greater in alpine pasture. Silage was only distributed in the indoor system and was composed of 17 g·kg^{-1} TEP, 13 g·kg^{-1} NTP, and 5 g·kg^{-1} CT.

3.2. In vitro incubation

Total gas production showed significant pairwise differences between farming systems and between genotypes, both in the test and blank fermenters (Tables 2 and 3). On average, the rumen fluid of calves from extensive systems (mid-elevation and alpine pastures) produced more gas than those from the intensive systems (indoor and lowland pasture) in both test and blank fermenters. Furthermore, rumen fluid of the crossbred calves produced more total gas in test and blank fermenters than Swiss Fleckvieh, while the Brown Swiss had intermediate values.

In the test fermenters, CH_4 volume and concentration were significantly greater in rumen fluid from the extensive systems. In the blank fermenters, CH_4 volume was greater with rumen fluid of calves from the extensive systems, while CH_4 concentration was greater in rumen fluid from the intensive systems. The global effect of genotypes was significant on CH_4 volume and concentration, except for CH_4 concentration in the blank fermenters. Rumen fluid from Swiss Fleckvieh exhibited lower gas volume as well as CH_4 volume and concentrations compared to the other breeds. No interactions of genotype with system occurred for any gas and CH_4 variables (Fig. 3).

Concerning NH_3 formation, the global effect of the farming system was statistically significant in the model for the test fermenters, and the global effect of the genotype was statistically significant in the model for the blank fermenters. However, none of the pairwise contrasts reached significance in the post-hoc analysis. Consistent with the methane findings, no genotype × system interaction was observed for ammonia.

In vitro organic matter digestibility was only significantly affected by the farming systems. The indoor system showed a significantly lower IVOMD compared to lowland pasture, without any effects of genotype.

Pre-fermentation pH was significantly affected by farming system ($p < 0.001$) and genotype ($p < 0.05$), with a significantly lower pH for the mid-elevation grassland and alpine systems (6.42 and 6.54, respectively), a medium pH for the indoor system (6.90) and a significantly greater pH for the lowland pasture system (7.11). The rumen fluid pH from the crossbred calves was lower (6.66) than the one from the Swiss Fleckvieh (6.81), while Brown Swiss did not show significant differences from other genotypes (6.75). Post-fermentation pH did not differ between farming systems or breeds, demonstrating the effectiveness of the buffer.

The time between sampling and analysis was included in the models

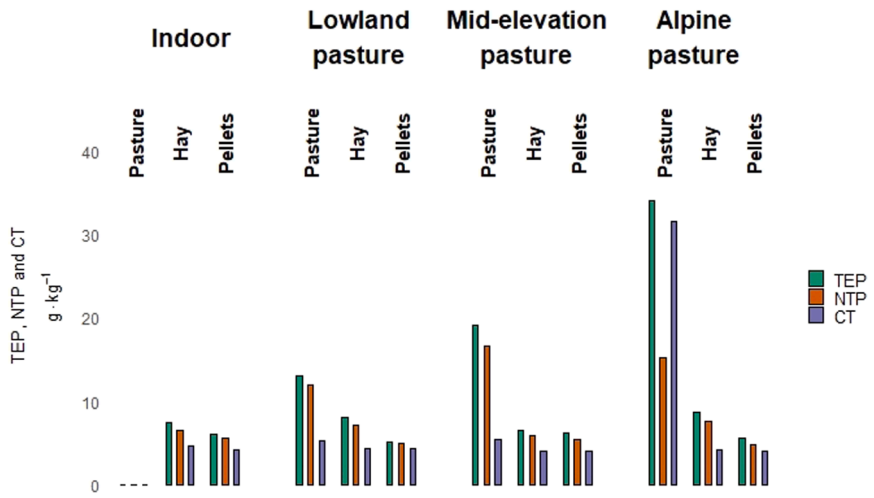

Fig. 2. Content of total extractable phenols (TEP), non-tannin phenols (NTP), and condensed tannins (CT) in feeds from the four experimental systems, sampled in the week preceding rumen fluid collection. Values are expressed as g/kg of dry matter.

Table 2
Effects of farming system and genotype on gas production and IVOMD after 24 h incubation with standard hay (test fermenters). Results include p-values from an ANOVA-like analysis, estimated marginal means for each categorical level ± standard error, and model fit metrics. BS: Braun Swiss, L × BS: Limousin × Brown Swiss crossbred, SF: Swiss Fleckvieh, RMSE: root-mean-square error. Note: calves from the mid-elevation system were not fasted prior to rumen fluid sampling.

Factor	Level	Total gas (ml·gDM^{-1})	CH_4 (ml·gDM^{-1})	CH_4/total gas (ml·100ml^{-1})	NH_3 (mmol·l^{-1})	IVOMD (g·kg^{-1})
System	Indoor	$221^{a} \pm 6.3$	$31.4^{a} \pm 1.8$	$14.1^{a} \pm 0.5$	13.0 ± 1.0	$638^{a} \pm 4.5$
	Lowland pasture	$231^{a} \pm 4.8$	$35.2^{a} \pm 1.5$	$15.2^{a} \pm 0.4$	14.7 ± 0.9	$652^{b} \pm 3.6$
	Mid-elevation pasture	$285^{b} \pm 5.8$	$50.1^{b} \pm 2.1$	$17.5^{b} \pm 0.4$	15.1 ± 0.9	$645^{ab} \pm 3.2$
	Alpine pasture	$276^{b} \pm 8.5$	$49.3^{b} \pm 3.2$	$17.8^{b} \pm 0.6$	12.0 ± 1.1	$656^{ab} \pm 5.0$
Genotype	BS	$250^{ab} \pm 4.5$	$40.1^{ab} \pm 1.5$	$15.9^{ab} \pm 0.3$	12.8 ± 0.7	646 ± 2.8
	L × BS	$260^{b} \pm 4.6$	$43.6^{b} \pm 1.6$	$16.7^{b} \pm 0.3$	14.9 ± 0.8	652 ± 2.9
	SF	$245^{a} \pm 4.3$	$38.4^{a} \pm 1.4$	$15.6^{a} \pm 0.3$	13.3 ± 0.7	645 ± 2.8
P value	System	<0.001	<0.001	<0.001	0.028	0.029
	Genotype	0.057	0.039	0.038	0.092	0.137
	System × Genotype	0.750	0.681	0.618	0.518	0.924
Quality	pseudo-R^2	0.62	0.57	0.48	0.33	0.24
	RMSE	18.57	5.83	1.22	2.84	11.17

to control for potential bias due to sample storage duration. It had a significant effect on total gas production and CH_4 concentration from blank and test fermenters, and CH_4 production from the test fermenters. A negative relationship was observed between time and gas production or CH_4 concentration from the test fermenters within each system (Fig. 4). On average, gas production decreased by 7.7 ml per hour of transport or storage time, CH_4 production by 2.6 ml, and CH_4 concentration by 0.5 percentage point. However, the overall trend across all systems was slightly positive. Note that the estimated marginal means in Tables 2 and 3 are corrected for these time effects.

4. Discussion

4.1. Evaluation of the methodology

Testing long-term rather than short-term effects of feeds on rumen fermentation is important because the rumen microbiota may adapt to plant secondary compounds and develop resistance to their impact (Cardozo et al., 2004; Kapp-Bitter et al., 2021b; Khiaosa-ard et al., 2012). However, this is a challenge for in vitro systems and is not usually applied.

Batch tests like the Hohenheim gas test of Menke and Steingass (1988) were designed to evaluate feed property effects on rumen fermentation based on standardized rumen fluid samples. Batch systems are often used for screenings of potential forage plants (Jayanegara et al., 2011; Soliva et al., 2008) but they are rarely used to compare different animals based on standardized feeds (Li et al., 2022). However, the latter is what the present study did. We compared rumen fluid from 48 individual calves of different genotypes that had grazed different pastures, in an Hohenheim gas test setting, where the test feed was one standardized hay. That also implied that the effects tested were long-term effects of genotype and feeding history of the calves, rather than immediate short-term effects of the feed in the batch system.

The approach came along with challenges. One was the proper and standardized sampling of rumen fluid by intubation from calves. A further challenge of the present study was that the sites were at different distances from the laboratory, which implied different time lags between sample drawing and the start of incubation. The third challenge was that the effect of standard feed during incubation might mask the effects of the feeding history before it. The success of the approach can only be assessed by the results.

The significant effects of system and genotype on gas production and methane formation found in the current study indicate that long-term effects remained detectable. The clear effect of transport time on the results shows that the immediacy of incubation matters. However, the review of Yáñez-Ruiz et al. (2016) concluded that the gas production was weakly affected until 350–500 min. This was confirmed by our observation in the present study and a preliminary study (unpublished), which showed that a delay of 480 min reduced total gas production from blank fermenters by approximately 12.5 %. Despite being rarely observed in other studies, the present study highlighted a significant effect of storage time, which needs to be accounted for in the statistical models. Thus, the inclusion of storage time as a covariate allowed us to account for this effect and improve the comparability of estimated

Table 3
Effects of farming system and genotype on gas production after 24-hour incubation without standard hay (blank fermenters). Results include p-values from an ANOVA-like analysis, estimated marginal means for each categorical level ± standard error, and model fit metrics. BS: Braun Swiss, L × BS: Limousin × Brown Swiss crossbred, SF: Swiss Fleckvieh, RMSE: root-mean-square error. Note: calves from the mid-elevation system were not fasted prior to rumen fluid sampling.

Factor	Level	Total gas (ml)	CH_4 (ml)	CH_4/total gas (ml·100ml^{-1})	NH_3 (mmol·l^{-1})
System	Indoor	4.71^{a} ± 0.7	0.88^{a} ± 0.2	2.57^{b} ± 0.3	17.0 ± 1.3
	Lowland pasture	4.65^{a} ± 0.5	0.83^{a} ± 0.1	2.43^{b} ± 0.2	17.6 ± 1.0
	Mid-elevation pasture	15.67^{b} ± 1.6	3.39^{b} ± 0.5	1.00^{a} ± 0.1	17.4 ± 0.9
	Alpine pasture	12.38^{b} ± 1.9	2.71^{b} ± 0.6	1.28^{a} ± 0.1	14.0 ± 1.1
Genotype	BS	7.96^{ab} ± 0.7	1.61^{ab} ± 0.2	1.72^{a} ± 0.1	16.0 ± 0.8
	L × BS	9.49^{b} ± 0.8	1.98^{b} ± 0.2	1.54^{a} ± 0.1	18.4 ± 0.9
	SF	6.96^{a} ± 0.6	1.30^{a} ± 0.2	1.79^{a} ± 0.1	15.1 ± 0.7
P value	System	<0.001	<0.001	<0.001	0.076
	Genotype	0.042	0.049	0.180	0.008
	System × Genotype	0.626	0.571	0.733	0.122
Quality	pseudo-R^2	0.71	0.65	0.68	0.41
	RMSE	3.61	0.9	0.41	3.05

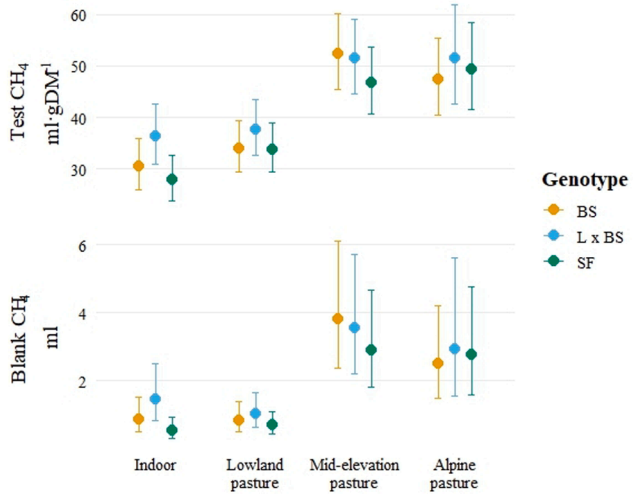

Fig. 3. In vitro methane production from the test (ml.gDM-1) and the blank (ml) fermenters, per system and genotype, after 24 hours of incubation. Genotypes are Brown Swiss (BS, dairy), Limousin × Brown Swiss (L × BS, crossbreed), and Swiss Fleckvieh (SF, dairy-oriented dual purpose).

marginal means between farming systems. Fermentations were initiated as soon as possible after sampling to limit the overall loss of microbial activity, rather than introducing a standardized delay across all samples. Other conservation methods, such as refrigerating or freezing the inoculum, can also be used, but they may delay initiation of the fermentation, kill bacteria, and modify the microbial communities (Prates et al., 2010). We conclude that intubation is a valid option for the assessment of animal effects on rumen fermentation in in vitro studies (Li et al., 2022), which can also be applied to calves or youngstock. However, currently the data can only serve for relative comparison of trends or potentials. For the deduction of absolute units of, for example, methanogenesis, a calibration against other methodological approaches is required.

4.2. *A strong effect of the grass-based system on the fermentation*

Previous studies have reported a negative correlation between grassland botanical diversity and methane emissions. Therefore, it was unexpected that the alpine pasture-based system exhibited the highest methanogenic potential in the present study. A possible explanation lies in the composition of the grasslands compared: while earlier studies often involved simplified or sown mixtures selected for their anti-methanogenic properties (Carmona-Flores et al., 2020; Hassan et al., 2025; Loza et al., 2021), we examined more spontaneous grasslands indirectly selected for their nutritive values. Macheboeuf et al. (2014) and Jayanegara et al. (2011) identified spontaneous species from montane grasslands with methane-reducing potential, attributed to their phenolic compounds and tannins. Although our alpine and mid-elevation pastures also had higher phenol and tannin contents, this was not associated with reduced gas production. However, these and many other studies evaluated the short-term effects of herbs in vitro. Grazing implies a long-term adaptation of the rumen microbiome to the plant secondary compounds, which can mitigate their effects (Khiaosa-ard et al., 2012; Smith et al., 2005). Our results agree with previous studies in adult ruminants, showing that the effects of methane inhibitors are mainly short-lived (Costigan et al., 2024; Kapp-Bitter et al., 2021b), though they may exert longer-lasting influences when exposure occurs early in life, by shaping the rumen microbiota (Meale et al., 2021).

Such an adaptation of the calves to the mountain pastures may explain why the pastures rich in phenols and tannins did not mitigate methane formation. However, for the fact that in vitro gas production and methanogenesis even increased, another mechanism must be held responsible. As a hypothesis, the rumen of calves on more fibrous pastures may have adapted to a greater need for fibre degradation by promoting fibrolytic bacteria, which then, during the in vitro test, had a stronger fibrolytic capacity, leading to higher fermentation rates and methane formation.

Previous in vivo studies have also found inconsistent effects of pasture composition on methane production (Pelve et al., 2012; Vargas et al., 2022), and partly undesired results of multispecies swards (Jonker et al., 2019; Loza et al., 2021). For grazing calves, Cristobal-Carballo et al. (2021) reported no long-lasting dietary effects on methanogenesis or any metabolites of ruminal fermentation.

According to our results, comparing indoor and outdoor systems may not be meaningful in itself, as the indoor and lowland pasture-based systems showed similar gas emissions, despite different diets. This finding is consistent with those from Cameron et al. (2018), who reported differences between grass-based and total mixed ration-based systems, but no significant differences between indoor and outdoor systems when both were based on fresh grass. These observations suggest that the nature of the diet (e.g. grass vs. total mixed ration) may be more influential than the housing system. Moreover, in our study, the lowland pasture-based calves were only able to graze for 611 h between 4 and 6 months of age due to a particularly rainy spring. This limited grazing time may have further contributed to the similarity between this system and the indoor one in terms of feed, and helps explain why it appeared closer to the indoor than to the more extensive outdoor systems in terms of gas emissions.

Feeding conditions influence rumen development (Klein et al., 1987) and consequently body weight, as reflected by the significant differences observed between systems. As these factors are inherent to the farming systems, body weight should be considered part of the system effect rather than an independent explanatory variable. Further research would be needed to disentangle the respective roles of grass-based diet, rumen development stage, and microbiota composition.

One limitation for the interpretation of our results is that the calves from the mid-elevation pasture system were not fasted before rumen

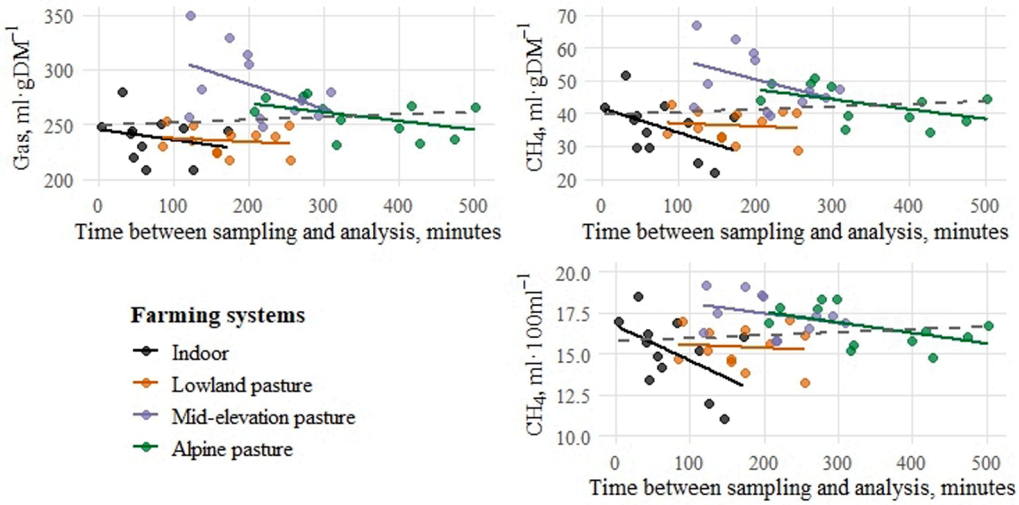

Fig. 4. Relationship between processing time lag and in vitro fermentation parameters. The figure shows total gas production (ml·gDM^{-1}), absolute methane production (ml·gDM^{-1}), and relative methane production (ml·100ml^{-1} total gas) as a function of the time elapsed between on-farm rumen fluid sampling and the beginning of the in-lab fermentation. Each point represents a single fermentation run, with colors indicating the system. Solid lines show the linear regression for each system, while the dashed line represents the overall linear regression across all systems.

fluid sampling, which may have contributed to the higher gas production observed. The literature on this topic is limited, as most studies do not sample rumen fluid as early as one hour after feeding. Ramos-Morales et al. (2014) reported no significant effect of fasting on ruminal ammonia concentration or the abundance of methanogenic archaea, whereas Maccarana et al. (2016) reported increased gas and methane post-feeding, though without specifying the sampling time. Importantly, in our study, the calves from the alpine pasture system were properly fasted and showed similar results to those from the mid-elevation pasture system, suggesting that the lack of fasting alone does not fully explain the observed differences between the intensive systems (indoor and lowland pasture) and the extensive systems (mid-elevation and alpine pastures). Nevertheless, this factor may have contributed to increased variability and should be considered when interpreting the results, although there is no evidence to suggest that it would have significantly altered the main conclusions of this study.

A possible explanation for similarities between the intensive systems (indoor and lowland pasture) and between the extensive systems (mid-elevation and alpine pastures) would be that the raising farm (from 3 weeks to 3 months of age) influenced the calves' rumen microbiota, thereby affecting gas production. Calves from the indoor and lowland pasture systems were raised on the same farm and showed lower gas emissions, whereas those from the mid-elevation and alpine pasture systems came from a second farm. To our knowledge, no study has directly compared the rumen microbiota of calves fed the same diet but raised on different farms. While diet is a known driver of microbial composition (Dill-McFarland et al., 2019), we standardized feeding across farms to minimize this effect. We could not control the environmental microbiome during this raising period, but Henderson et al. (2015) reported similar rumen microbiota across continents, suggesting that calves raised within 60 km of each other are unlikely to develop significantly different microbial profiles.

4.3. A significant but low effect of breed

The effect of breed on enteric gas production is still largely debated. Many studies report non-significant differences (De Mulder et al., 2018; e.g. Duthie et al., 2017), and when effects are significant, they are often attributed more to differences in dry matter intake than to genetic differences per se (Boadi and Wittenberg, 2002).

Wang et al. (2018) found that rumen fluid from beef cattle produced more methane, a lower CH_4/total gas ratio, and less ammonia than those from dairy cattle. Similarly, in our study, rumen fluid from crossbred beef × dairy calves yielded significantly greater gas and CH_4 productions, and a non-significant trend toward greater NH_3, compared to the dairy-oriented dual-purpose breed calves.

To assess sustainability, emissions should ideally be expressed per kilogram of meat produced. Our crossbred calves produced 14 to 52 % more CH_4 in vitro than dual-purpose calves; thus, they would have to produce at least 14 % more meat to be considered more environmentally efficient, if in vitro CH_4 emissions are linearly correlated to in vivo emissions as observed in Jayanegara et al. (2012). Nevertheless, while CH_4 production measured in vitro per unit of organic matter shows a reasonable correlation with in vivo results (Yáñez-Ruiz et al., 2016), such comparisons remain approximative without direct in vivo emission data.

Importantly, the effect of genotypes appears modest compared to the effect of the farming system. For example, CH_4 production was 49 % greater in samples from extensive systems compared to intensive ones, whereas the breed effect reached only 14 %. Additionally, breed-based rankings of gas and NH_3 production can change with incubation duration, as shown by Kang et al. (2024) after 3 to 24 h incubation, suggesting methodological factors can influence apparent breed effects.

5. Conclusion

This study is among the first to compare the methanogenic potential of calves raised under different grass-based systems. We adapted a conventional in vitro batch test to enable an accurate comparison of gas production between animals. Our results underline the importance of including the time between sampling and analysis in statistical models, to account for differences in farm-to-laboratory transport time. The findings revealed a strong effect of the farming system on in vitro gas, methane, and ammonia production, and a significant but less pronounced effect of breed. The more intensive systems (indoor and lowland pasture) were generally associated with lower production of total gas, methane, and ammonia than extensive systems (mid-elevation and alpine pastures). These findings provide insights into the environmental impact of different farming strategies, which are stronger than reported effects from on-station trials. However, the presented on-site methodology and the target system (grazing young calves) still lack broader experience and research literature to contextualize and interpret the

data. In that sense, the current study serves as pioneer work.

CRediT authorship contribution statement

Geoffrey Mesbahi: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Florian Leiber:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Andrea K. Steiner:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Markus Leubin:** Investigation. **Susann Thüer:** Investigation. **Marie T. Dittmann:** Writing – review & editing, Investigation, Funding acquisition, Data curation. **Amarante Vitra:** Writing – review & editing, Investigation, Data curation. **Jessica Werner:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We warmly thank Melissa Terranova for coordination at Agrovet-Strickhof, and the farmers Flavio Ferrari, Marina Fliri, and Pascal Nägele for animal care. We are very grateful to Anna Neujahr, Elodie Mayoraz, Cem Baki, and Vladyslav Teslia for their assistance with sample collection, and to Manuela Helbing for her support in conducting the Hohenheim gas tests. This work has received funding from the European Union's Horizon Europe research and innovation program under the grant agreement No 10105960 (Re-Livestock project).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.livsci.2026.105968.

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