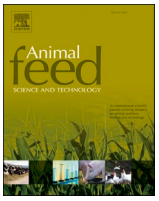
Animal Feed Science and Technology 332 (2026) 116611

Contents lists available at ScienceDirect

Animal Feed Science and Technology

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



Modelling methane emissions from ruminant diets with variable forage-to-concentrate ratios and retention times – An *in vitro* evaluation

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ARTICLE INFO

Keywords:
Forage-to-concentrate ratio
Methane production
Modelling
Nordic ruminant diets
Automated *in vitro* system

ABSTRACT

This study aimed to improve the predictive accuracy of *in vitro* models for estimating *in vivo* methane (CH_4) emissions in Nordic dairy systems by evaluating five forage-to-concentrate (F:C) ratios and incorporating a modelling approach based on ruminal mean retention time (MRT). The tested ratios included 100:0 (100 F), 80:20 (80 F), 60:40 (60 F), 40:60 (40 F), and 20:80 (20 F), where 100 F consisted solely of grass silage, and the remaining diets incorporated barley grain and rapeseed meal as concentrate. All diets were balanced for crude protein (20 % DM), but ether extract and neutral detergent fiber content decreased as concentrate levels increased. To improve the biological relevance of *in vitro* results, CH_4 production was corrected using a ruminal MRT model to better simulate *in vivo* conditions. Higher concentrate inclusion linearly increased ($P < 0.001$) total gas and predicted *in vivo* CH_4 production. However, after applying MRT adjustments, the modified model reduced the variation in CH_4 predictions across F:C ratios, resulting in values that more closely reflected expected *in vivo* emissions. The pH declined ($P < 0.001$) at lower F:C ratios. Organic matter degradability (OMD) followed a quadratic pattern ($P < 0.001$), peaking in 60 F and 40 F diets and decreasing in 100 F and 20 F. While total volatile fatty acid concentrations were unaffected by F:C ratio, acetate proportion declined linearly ($P < 0.001$) as concentrate increased, whereas isobutyric and butyric acid proportions rose. Overall, these findings support the application of MRT-adjusted models to enhance the alignment between *in vitro* predictions and *in vivo* CH_4 emissions.

1. Introduction

Animal production is a significant contributor to greenhouse gas emissions, particularly methane (CH_4) and nitrous oxide, which arise from rumen fermentation and manure management (Hristov et al., 2013). Global anthropogenic greenhouse gas emissions

Abbreviations: CH_4, methane; F:C, forage-to-concentrate; MRT, mean retention time; 100 F, 100:0 forage-to-concentrate ratio; 80 F, 80:20 forage-to-concentrate ratio; 60 F, 60:40 forage-to-concentrate ratio; 40 F, 40:60 forage-to-concentrate ratio; 20 F, 20:80 forage-to-concentrate ratio; DM, dry matter; H_2, hydrogen; OM, organic matter; VFA, volatile fatty acids; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; CO_2, carbon dioxide; iNDF, indigestible NDF; NH_3-N, ammonia-N; DMD, dry matter degradability; OMD, organic matter degradability; TGP, total gas production; CH_4/TGP, ratio of predicted *in vivo* CH_4 to TGP; BCVFA, branched-chain VFA; A:P, acetate to propionate.

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https://doi.org/10.1016/j.anifeedsci.2025.116611
Received 2 June 2025; Received in revised form 30 September 2025; Accepted 18 December 2025
Available online 20 December 2025

associated with milk consumption are projected to triple by 2050 (Ma et al., 2025). As a result, livestock production systems are increasingly focused on reducing their environmental impact while maintaining economic viability. Sustainable agriculture aims not only to enhance the production of nutrient-dense food but also to minimize emissions and improve efficiency, all while ensuring farm profitability (Richardson et al., 2023).

One of the most effective CH_4 mitigation strategies is dietary manipulation, which influences ruminal fermentation pathways, microbial hydrogen (H_2) flow, and substrate fermentability (Beauchemin et al., 2020). While concentrate-rich diets improve dairy cow productivity and feed efficiency, their high cost and contribution to food-feed competition pose economic and sustainability challenges (Ormston et al., 2025). In addition, possible metabolic consequences of diets rich in non-fibrous carbohydrates include ruminal acidosis, reduced milk fat and shorter productive life of animals (Ribeiro Pereira et al., 2015). Furthermore, high- concentrate diets may lead to increased fermentable organic matter (OM) in manure, potentially elevating CH_4 emissions from manure decomposition (Hristov et al., 2013). The economic viability of production systems with high levels of concentrate is questionable in climates more conducive to forage-based production (Ribeiro Pereira et al., 2015). Increasing forage proportion in diets can mitigate the aforementioned concerns by lowering feeding expenses and reducing reliance on resource-intensive concentrates, though often at the expense of production levels (Ormston et al., 2025).

The diet composition directly affects ruminal H_2 availability and CH_4 emissions. According to stoichiometric principles, acetate and butyrate formation generates H_2, promoting CH_4 production, whereas propionate formation acts as a competitive pathway by consuming H_2 (Moss et al., 2000). Fiber-rich diets enhance H_2 availability, while starch-rich diets reduce H_2 release (Aguerre et al., 2011; Hristov et al., 2013). Additionally, differences in feed degradability and chemical composition influence volatile fatty acid (VFA) profiles, shaping CH_4 emissions. Microbial protein assimilation and nitrogen metabolism can also either contribute to or mitigate H_2 availability (Knapp et al., 2014).

In vitro systems have been developed to measure ruminant feedstuff quality and to screen rumen fermentation characteristics (Serment et al., 2016). However, *in vitro* fermentation models face limitations when evaluating CH_4 production under different dietary conditions. Danielsson et al. (2017) reported reduced reliability of an automated *in vitro* system with increasing concentrate inclusion, potentially due to the high fermentability of concentrates and the closed nature of the system, which does not account for substrate-specific ruminal retention times. Furthermore, CH_4 production from *in vitro* studies varies across regions due to differences in the basal diets of donor animals, affecting the composition of rumen fluid inoculum. For example, studies using the same substrate have reported variable CH_4 outputs depending on the source of the inoculum (Kim et al., 2018).

This study aimed to evaluate the effects of varying forage-to-concentrate (F:C) ratios and modeled mean retention times (MRT) in the rumen on CH_4 production, using an automated *in vitro* gas production system. By using different dietary compositions and rumen passage rates, we sought to improve the biological relevance and predictive accuracy of *in vitro* models for estimating *in vivo* CH_4 emissions under Nordic feeding conditions. To achieve this, we incorporated a modelling approach that adjusts *in vivo* CH_4 predictions based on diet-specific MRT values, thereby better reflecting the fermentation dynamics observed *in vivo*—especially in high-concentrate diets where feed particles pass more rapidly through the rumen. We hypothesized that (1) increasing concentrate inclusion would alter CH_4 production due to the high fermentability of the substrate, and (2) adjusting the *in vitro* model to reflect diet-specific MRT would improve its ability to predict CH_4 production, particularly for high-concentrate diets.

2. Materials and methods

2.1. Ethical compliance

All animal procedures were conducted in accordance with Swedish laws and regulations regarding EU Directive 2010/63/EU on animal research and were approved by the Swedish Ethics Committee on Animal Research (Dnr A 17/2016 and A 33/2016, Umeå, Sweden). The experiment was conducted in the laboratory of the Department of Applied Animal Science and Welfare, Umeå, Sweden.

2.2. Substrates, treatments, and experimental design

A fully automated *in vitro* gas production system (Ramin and Huhtanen, 2012) was used to evaluate the effects of different F:C ratios on CH_4 production and ruminal fermentation. Five experimental diets were formulated with varying F:C ratios: 100:0 (100 F), 80:20 (80 F), 60:40 (60 F), 40:60 (40 F), and 20:80 (20 F) on a dry matter (DM) basis. The diets were composed of grass silage, barley grain

Table 1
Chemical composition of the dietary ingredients used for the experimental diets *in vitro*.

	g/kg of fresh matter	g/kg of DM					
Dietary ingredient	DM	Ash	CP	EE	NDF	iNDF	Starch
Barley grain	905	25.3	95.0	17.1	152	28.5	613
RSM	924	76.6	368	24.1	321	122	22.4
Grass silage[a]	315	77.9	203	37.5	420	29.8	8.13

DM: dry matter. CP: crude protein. EE: ether extract. NDF: neutral detergent fiber. iNDF: indigestible NDF. RSM: rapeseed meal.
[a] Grass silage characteristics: NH_3-N (53.8 g/kg of N), lactic acid (108 g/kg DM), acetic acid (18.9 g/kg DM), propionic acid (4.13 g/kg DM), butyric acid (< 0.01 g/kg DM), pH = 3.79.

(*Hordeum vulgare*), and rapeseed meal (*Brassica napus*), with their chemical compositions detailed in Tables 1 and 2. The silage was made from first-cut perennial leys of timothy (*Phleum pratense*).

All diets were balanced (isonitrogenous) to match the crude protein (CP) content of the grass silage, ensuring a uniform CP content of 203–204 g/kg DM. The ether extract (EE) content decreased as the proportion of concentrate increased, ranging from 37.5 to 23.3 g/kg DM. Similarly, the neutral detergent fiber (NDF) content was highest in forage-rich diets, ranging from 260 to 420 g/kg DM.

A day before incubation, dried and ground (mill size 1.0 mm; Retsch SM2000; Rheinische, Haan, Germany) experimental substrates were weighed into sterile 250 mL serum bottles (Schott, Mainz, Germany). The amount of each substrate in the bottle was adjusted according to the proportions of the experimental diets with final weights of 1.0 g for each diet.

The incubations were performed in two *in vitro* gas production runs conducted in different weeks. Each run included six blank bottles (no substrate) and four bottles per treatment.

2.3. Animals and in vitro incubation

Rumen content was obtained from two cannulated Nordic Red cows (682 ± 55 kg body weight; 77 ± 14 DIM). Cows were fed a mixed ration consisting of second-cut grass silage from timothy (Ara 11, Lantmännen, Sweden; 600 g/kg DM), concentrate (Komplett Xtra 200, Lantmännen, Sweden; 391 g/kg DM), and a mineral supplement (Mixa Intensiv mg, Lantmännen, Sweden; 9 g/kg DM). The diet was provided twice daily *ad libitum*.

Rumen content was collected two hours after morning feeding into pre-warmed thermal flasks that had been flushed with carbon dioxide (CO_2). Within 15 min, the flasks were transported to the laboratory, where the rumen contents (pH 6.37 ± 0.122, mean ± SE) were pooled and filtered through four layers of cheesecloth to obtain rumen fluid while being continuously flushed with CO_2.

The rumen fluid was then buffered with a mineral solution (20:80 v/v; Menke and Steingass, 1988) and supplemented with 2 g of peptone (Merck, Darmstadt, Germany). Under a continuous CO_2 flush, each serum bottle was filled with 60 mL of buffered rumen fluid and incubated in a temperature-controlled water bath at 39 °C with gentle shaking (40 RPM). Gas production was measured according to the method described by Cone et al. (1996).

2.4. Chemical analysis of dietary ingredients

Grass silage and concentrate were analyzed for DM, ash, CP, EE, NDF, indigestible NDF (iNDF), and starch. The samples were oven dried at 105°C for 16 h to determine DM content, followed by ash quantification through combustion at 500°C for 4 h (Horwitz, 2000). Total nitrogen (N) was assessed by the Kjeldahl method, with CP calculated as total N × 6.25. Ether extract content was determined via ether extraction and HCl hydrolysis following AOAC method 954.02 (Horwitz, 2000). Finally, starch concentration was analyzed according to the method of Larsson and Bengtsson (1983).

Silage fermentation characteristics were evaluated by measuring pH, fermentation acid composition, and ammonia-N (NH_3-N) concentration. Before the analysis, frozen silage samples were thawed and pressed to extract juice. Afterwards, the extracted juice was diluted (1:1) with distilled water. Lactic acid and VFA concentrations were determined following the method of Ericson and André (2010), while NH_3-N was measured using a Kjeltec 2100 Distillation Unit (Foss Analytical Ltd.). The silage DM content was corrected for volatile losses using the equation described by Huida et al. (1986).

2.5. Sampling and in vitro degradability measurements

Gas was sampled at 2, 4, 8, 24, and 48 h of incubation using a gas-tight syringe (Hamilton, Bonaduz, Switzerland). Methane concentration was analyzed using gas chromatography (Thermo Scientific™ TRACE 1300™ Series Gas Chromatograph, Thermo Fisher Scientific S.p.A. Milan, Italy) equipped with a thermal conductivity detector. Samples (0.5 mL) for VFA and NH_3-N analysis were collected from two replicates per treatment in each run after 48 h of incubation. Volatile fatty acid concentrations were determined using high-performance liquid chromatography (Ericson and André, 2010) with a Waters Acquity UPLC system (Waters, Milford, MA, USA) equipped with a BEH C18 reverse-phase column (2.1 × 100 mm, 1.7 μm). The analysis was performed at 45°C using a gradient elution of formic acid in water and acetonitrile, with a flow rate of 0.4 mL/min and detection at 269 nm. The analytical method followed the procedure described by Puhakka et al. (2016). The NH_3-N concentration was determined using a continuous flow analyzer (AutoAnalyzer 3 HR; SEAL Analytical, Southampton, UK; Vaga et al. 2017) by measuring absorbance at 660 nm. Before analysis, the

Table 2
Dietary proportions (%) and chemical composition (g/kg DM) of the experimental diets incubated *in vitro*.

Diet	Silage proportion	Barley grain proportion	RSM proportion	CP	EE	Starch	NDF	iNDF
100 F	1.00	0.00	0.00	203	37.5	8.13	420	29.8
80 F	0.80	0.12	0.08	203	34.0	81.9	380	37.0
60 F	0.60	0.24	0.16	204	30.4	156	340	44.2
40 F	0.40	0.36	0.24	204	26.9	229	300	51.5
20 F	0.20	0.48	0.32	204	23.3	303	260	58.7

RSM: rapeseed meal. CP: crude protein. EE: ether extract. NDF: neutral detergent fiber.
The forage-to-concentrate ratios of experimental diets were 100:0 (100 F), 80:20 (80 F), 60:40 (60 F), 40:60 (40 F), and 20:80 (20 F).

samples were thawed and centrifuged at 12,500 × g for 10 min. A 0.1 mL aliquot of the supernatant was then diluted 1:25 with MQ water and transferred to test tubes for analysis.

At the end of the 48-hour incubation, pH was recorded (Metrohm Ltd., Herisau, Switzerland) and fermentation was terminated by placing the bottles on ice.

In vitro degradability was assessed after 48 h of incubation, following Fant and Ramin (2024). Briefly, residues were transferred to pre-weighed 11 μm nylon bags (Sefar AG, Switzerland), with excess liquid filtered out. To determine dry matter degradability (DMD) and OM degradability (OMD), residues were boiled in a neutral detergent solution with heat-stable α-amylase and sodium sulfite to remove microbial material (Makkar et al., 1995; Van Soest and Robertson, 1985). Bags were then dried at 60°C for 48 h and weighed to calculate *in vitro* DMD using the following equation:

DMD (g/kg) = [Incubated DM (g) – NDF residue corrected for blank (g)] / 1000 × Incubated DM (g).

In vitro OMD was measured by combusting the incubation residues (excluding bags) for four hours at 500°C and calculated using the following equation:

OMD (g/kg) = [Incubated OM (g) – NDF residue corrected for ash and blank (g)] / 1000 × Incubated OM (g).

2.6. Prediction of in vivo CH_4 production

In vivo CH_4 production was predicted from *in vitro* gas production data following the methodology described by Ramin and Huhtanen (2012). The accumulated CH_4 production (mL) at each measured time interval (0.2 h) was calculated as:

$$V_{CH4}\ (mL) = V_{HS}\ (mL) \times CH_4\ (mL/mL) + V_{GP}\ (mL) \times A \times CH_4\ (mL/mL),$$

where V_{CH4} represents total CH_4 output at each time interval; V_{HS} is the headspace volume; CH_4 is the CH_4 concentration in the headspace; V_{GP} is the total gas volume produced; and A is the ratio of CH_4 concentration in the outflow gas to that in the headspace. Coefficient A (0.55) was predicted using a mechanistic model (Ramin and Huhtanen, 2012).

The CH_4 concentration at each 0.2-hour interval was estimated by fitting a logarithmic regression to the measured CH_4 values at five time points (2, 4, 8, 24 and 48 h). The total gas and CH_4 production data at each 0.2-hour time point were then fitted to a two pool Gompertz model (Schofield et al., 1994). The kinetic parameters of total gas and CH_4 release at each time point were predicted using the NLIN procedure in SAS version 9.4 (SAS Institute Inc., 2025):

$$V_t = V_1 \times Exp\{- Exp[1 - k_1 \times (t - L_1)]\} + V_2 \times Exp\{- Exp[1 - k_2 \times (t - L_2)]\},$$

where V_t is the measured total gas or CH_4 volume at time t; V_1, k_1, and L_1 are, respectively, the asymptotic cumulative gas production (mL/g of DM), rate (1/h), and lag time (h) parameters for the first pool, which represents the rapidly degradable substrates (fast); V_2, k_2, and L_2 are the corresponding parameters for the second pool, which represents the slowly degradable substrates (slow); and t is incubation time.

The estimated kinetic parameters were subsequently used in a dynamic, mechanistic two-compartment rumen model (Huhtanen et al., 2008) with modifications by Ramin and Huhtanen (2012). This model assumes different ruminal MRT and compartment-specific MRTs (CMRT1, CMRT2) for the first (rapid) and second (slow) compartments, respectively. Simulations were performed using three MRT scenarios:

1. 50 h MRT (20 h in CMRT1, 30 h in CMRT2) – representing dairy cows at maintenance intake.
2. 35 h MRT (14 h in CMRT1, 21 h in CMRT2) – representing dairy cows consuming approximately 20 kg DM/day (Krizsan et al., 2010).
3. Modified MRT – adjusted for differences in passage rate due to varying dietary F:C ratios.

Since concentrates pass through the rumen more rapidly than forages (Huhtanen et al., 2015), we implemented a modified MRT that accounts for dietary F:C ratio using the mechanistic Karoline model (Ramin and Huhtanen, 2015). As concentrate proportion

Table 3
Parameters used for the "modified" mean rumen retention time (MRT, in hours) based on estimates derived from the Karoline model (Huhtanen et al., 2015).

Treatments	MRT (h)	CMRT1 (h)	CMRT2 (h)
100 F	50.0	15.0	35.0
80 F	46.3	13.0	33.3
60 F	42.5	11.1	31.5
40 F	38.8	9.30	29.5
20 F	35.0	7.70	27.3

MRT (h) = mean retention time. CMRT1 = compartment MRT rapid pool. CMRT2 = compartment MRT slow pool.
The forage-to-concentrate ratios of experimental diets were 100:0 (100 F), 80:20 (80 F), 60:40 (60 F), 40:60 (40 F), and 20:80 (20 F).

increased, MRT decreased, with a proportionally greater reduction in CMRT1 (rapid pool) than in CMRT2 (slow pool) (Table 3). The estimated passage rates (kp, h^{-1}) used in our analysis are given in Table 4.

The final predicted *in vivo* CH_4 production (mL/g of DM), was calculated as the proportion of asymptotic CH_4 production multiplied by the asymptotic CH_4 production (mL/g of DM) as described by Ramin and Huhtanen (2012).

2.7. Statistical analysis

Data were statistically analysed using the MIXED procedure of SAS® 9.4 (SAS Institute Inc., 2025) according to the following model:

$$Y_{ijk} = \mu + T_i + R_j + B_k + e_{ijk}$$

where Y_{ijk}= observation, μ = population mean, T_i = treatment effect (i = 5), R_j = run effect (j = 2), B_k = bottle effect (k = 30) and e_{ijk} = residual error. T_i and R_j were considered fixed effects while B_k was treated as a random effect. To evaluate the effects of different F:C ratios, linear and quadratic orthogonal contrasts were performed. Differences between treatments were assessed as significant if $P \leq 0.05$, whereas a tendency toward significant was considered if $0.05 < P \leq 0.10$.

3. Results

3.1. Total gas and predicted in vivo CH_4 production

The treatment effects on *in vitro* total gas production (TGP) and predicted *in vivo* CH_4 production are presented in Table 4, and the values employed for the modified MRTs are presented in Table 3. The asymptotic gas production increased with higher concentrate levels ($P = 0.002$). Predicted TGP using MRT of 35 h, 50 h, and modified MRTs followed a similar trend ($P < 0.001$). Fermentation rates

Table 4
The effect of different F:C ratios on predicted *in vivo* total gas and CH_4 production parameters using different mean rumen retention times.

	Diet						*P*		
Item	100 F	80 F	60 F	40 F	20 F	SEM	F:C	Lin	Quad
Gas production parameters									
V_1 (mL/g DM)	186	220	191	154	135	16.4	0.010	0.003	0.059
L_1 (h)	0.378	-0.173	-0.013	0.312	0.387	0.1330	0.008	0.163	0.003
K_1 (h^{-1})	0.076	0.083	0.089	0.362	0.558	0.0760	< 0.001	< 0.001	0.027
V_2 (mL/g DM)	94.2	61.2	95.7	145	169	19.10	0.008	0.001	0.077
L_2 (h)	3.60	9.94	4.97	3.28	2.05	1.500	0.005	0.037	0.036
K_2 (h^{-1})	0.023	0.030	0.040	0.056	0.073	0.0050	< 0.001	< 0.001	0.149
kd Gas (h^{-1}, 35 h)	0.071	0.090	0.119	0.154	0.195	0.0070	< 0.001	< 0.001	0.028
kd Gas (h^{-1}, 50 h)	0.072	0.093	0.125	0.166	0.213	0.0080	< 0.001	< 0.001	0.030
kd Gas (h^{-1}, Modified)	0.069	0.090	0.113	0.143	0.171	0.0100	< 0.001	< 0.001	0.240
Asymptotic Gas production (mL/g DM)	280	281	287	299	304	4.5	0.002	< 0.001	0.370
Total Gas production (mL/g DM, 35 h)	223	239	255	276	287	4.5	< 0.001	< 0.001	0.703
Total Gas production (mL/g DM, 50 h)	243	256	268	287	295	4.5	< 0.001	< 0.001	0.836
Total Gas production (mL/g DM, Modified)	240	248	258	274	280	4.6	< 0.001	< 0.001	0.909
CH_4 production parameters									
V_1 CH_4 (mL/g DM)	19.1	21.5	22.4	24.2	24.7	0.57	< 0.001	< 0.001	0.142
L_1 CH_4 (h)	1.73	1.49	1.37	1.38	1.33	0.053	< 0.001	< 0.001	0.013
K_1 CH_4 (h^{-1})	0.109	0.118	0.138	0.148	0.163	0.0050	< 0.001	< 0.001	0.840
V_2 CH_4 (mL/g DM)	27.0	30.1	31.2	31.9	30.9	0.76	< 0.001	< 0.001	0.004
L_2 CH_4 (h)	7.92	6.93	6.08	5.44	4.60	0.181	< 0.001	< 0.001	0.365
K_2 CH_4 (h^{-1})	0.027	0.028	0.030	0.032	0.034	0.0005	< 0.001	< 0.001	0.438
kd CH_4 (h^{-1}, 35 h)	0.052	0.056	0.063	0.068	0.076	0.0010	< 0.001	< 0.001	0.083
kd CH_4 (h^{-1}, 50 h)	0.055	0.059	0.066	0.071	0.080	0.0010	< 0.001	< 0.001	0.095
kd CH_4 (h^{-1}, Modified)	0.053	0.056	0.062	0.065	0.072	0.0010	< 0.001	< 0.001	0.094
Asymptotic CH_4 (mL/g DM)	46.0	51.5	53.6	55.9	56.6	1.32	< 0.001	< 0.001	0.026
Total CH_4 production (mL/g DM, 35 h)	33.3	38.8	41.3	43.9	44.8	1.09	< 0.001	< 0.001	0.030
Total CH_4 production (mL/g DM, 50 h)	37.7	43.4	45.9	48.4	49.0	1.19	< 0.001	< 0.001	0.027
Total CH_4 production (mL/g DM, Modified)	36.9	41.5	42.8	43.8	43.2	1.11	< 0.001	< 0.001	0.015
Total CH_4/TGP Ratio									
Total CH_4/TGP Ratio (35 h)	0.150	0.160	0.162	0.161	0.157	0.0030	0.017	0.065	0.006
Total CH_4/TGP Ratio (50 h)	0.156	0.168	0.170	0.171	0.167	0.0030	0.003	0.007	0.004
Total CH_4/TGP Ratio (Modified)	0.155	0.165	0.166	0.162	0.155	0.0030	0.014	0.754	< 0.001

The forage-to-concentrate ratios of experimental diets were 100:0 (100 F), 80:20 (80 F), 60:40 (60 F), 40:60 (40 F), and 20:80 (20 F). SEM: highest standard error of mean. Lin: linear effect of increasing dietary inclusion of concentrate. Quad: quadratic effect of increasing dietary inclusion of concentrate. V_1: volume for the first pool (rapid pool). k_1: rate for the first pool (rapid pool). L_1: lag for the first pool (rapid pool). V_2: volume for the second pool (slow pool). k_2: rate for the second pool (slow pool). L_2: lag for the second pool (slow pool). Estimated passage rates (kp, h^{-1}): 35 h MRT = 0.048; 50 h MRT = 0.033. Modified MRT: 100 F = 0.029, 80 F = 0.030, 60 F = 0.032, 40 F = 0.034, 20 F = 0.037.

(K_1, K_2) and the rate of gas production (kd Gas) also increased with increased concentrate inclusion ($P < 0.001$), suggesting a faster fermentation process.

Similarly, predicted *in vivo* CH_4 production increased with a higher proportion of concentrate, with asymptotic CH_4 production, predicted *in vivo* CH_4 production at 35 h, 50 h, and modified MRTs all rising as F:C decreased ($P < 0.001$). The rate of CH_4 production (kd CH_4) followed a similar pattern ($P < 0.001$).

The ratio of predicted *in vivo* CH_4 to TGP (CH_4/TGP) showed a slight increase as the concentrate proportion in the diet increased. However, in the modified MRT prediction, this trend was less pronounced, and the ratio remained more stable across diets. Notably, the CH_4/TGP ratio using the modified MRT for the highest concentrate diet (20 F) was similar to that of the high-forage diets. The linear effect of increased concentrate inclusion on CH_4/TGP ratio was significant when using 50 h MRT ($P = 0.007$), whereas using the modified MRT did not result in any linear effect ($P = 0.754$) but instead showed a quadratic effect ($P < 0.001$) on CH_4/TGP ratio.

3.2. *In vitro* degradability and fermentation parameters

In vitro degradability and fermentation parameters were influenced by the F:C ratio and the results are presented in Tables 5 and 6, respectively. The DMD and OMD showed a slight but significant increase with moderate concentrate inclusion (60 F and 40 F), peaking at 90.4 % and 91.4 %, respectively, before slightly declining at the highest concentrate level (20 F; quadratic effect: $P = 0.003$ for DMD, $P < 0.001$ for OMD).

While total VFA production (mmol/g DM) did not differ significantly, individual VFA profiles showed notable shifts. Acetate concentrations decreased linearly with increasing concentrate levels ($P < 0.001$), from 606 mmol/L in 100 F to 578 mmol/L in 20 F, while butyrate followed the opposite trend ($P < 0.001$), increasing from 106 to 130 mmol/L. Propionate levels remained relatively stable ($P = 0.263$) and there was no significant increases in propionate production by increasing the concentrate proportion in the diets (1.5 mmol/g DM for the 100 F diet and 1.54 mmol/g DM for the 20 F diet). Meanwhile, isobutyric and butyric acids increased significantly with higher proportions of concentrate ($P < 0.001$). The rumen fluid pH exhibited a minor decreasing trend ($P = 0.015$) with higher concentrate inclusion, but all values remained above 6.2. The NH_3-N concentrations increased with higher concentrate inclusion ($P < 0.001$), reaching 21.4 mg/dL in the 20 F diet.

4. Discussion

Incubating diets with varying F:C ratios in an automated *in vitro* system confirmed our hypotheses: 1) higher concentrate proportions alter CH_4 production, and 2) adjustments to the *in vitro* model system are necessary to account for different substrate retention times. Overall, the results highlight that adjusting for MRT helps *in vitro* models better reflect CH_4 emissions observed *in vivo*. Further, moderate concentrate inclusion (40F–60 F) optimizes degradability, whereas higher levels (20 F) promote protein degradation and shift the VFA profile towards butyrate and branched-chain VFA (BCVFA).

4.1. *Total gas and predicted in vivo CH_4 production*

The TGP clearly reflects the effect of the F:C ratio on fermentation dynamics. As the concentrate level increased, both the original and MRT-adjusted models predicted a steady rise in TGP, consistent with previous research (Azmi et al., 2020; Kim et al., 2018; Serment et al., 2016; Vera et al., 2025). Our data confirms the previously reported linear relationship between TGP and concentrate inclusion (Kim et al., 2018). The rate of gas production (kd Gas) increased with higher concentrate levels, reflecting a faster fermentation rate, though the TGP and rate of gas production are not necessarily causally linked.

The F:C ratio significantly influenced predicted *in vivo* CH_4 production, following a trend similar to TGP. Higher concentrate proportions increased CH_4 output, aligning with previous studies (Azmi et al., 2020; Kim et al., 2018; Serment et al., 2016; Vera et al., 2025). While CH_4 production is typically associated with fiber fermentation, rapidly digestible feeds can also contribute to significant CH_4 production during the early stages of fermentation due to their breakdown (Getachew et al., 2005b; Lee et al., 2003; Ribeiro Pereira et al., 2015). An increased availability of fermentable carbohydrates results in greater CH_4 production, owing to the need to dispose of excess reducing equivalents (Hristov, 2024). The reduced lag times observed in high-concentrate diets are consistent with the faster degradation of starch-rich substrates (Lee et al., 2003), which typically exhibit shorter lag phases.

Our results indicate a sustained increase in CH_4 production over time, with the CH_4/TGP ratio increasing significantly, likely due to the fermentation of less digestible components towards the end of the incubation period. The increase in the ratio of CH_4/TGP reflects a

Table 5
The effects of different F:C on *in vitro* degradability parameters.

	Diet						*P*		
Item	100 F	80 F	60 F	40 F	20 F	SEM	F:C	Lin	Quad
DMD (%)	88.9	90.0	90.4	90.4	89.8	0.36	0.009	0.023	0.003
OMD (%)	89.3	90.7	91.4	91.2	90.4	0.42	0.010	0.006	< 0.001

The forage-to-concentrate (F:C) ratios of experimental diets were 100:0 (100 F), 80:20 (80 F), 60:40 (60 F), 40:60 (40 F), and 20:80 (20 F).
SEM: highest standard error of mean. DMD: dry matter degradability. OMD: organic matter degradability. Lin: linear effect of increasing dietary inclusion of concentrate. Quad: quadratic effect of increasing dietary inclusion of concentrate.

Table 6
The effect of different F:C ratio on *in vitro* fermentation parameters.

	Diet						*P*		
Item	100 F	80 F	60 F	40 F	20 F	SEM	F:C	Lin	Quad
Total VFA mmol/g DM	6.07	6.58	6.05	6.32	6.47	0.270	0.492	0.468	0.909
VFA molar proportions (mmol/mol total VFA)									
Acetate	606	604	595	590	578	2.2	< 0.001	< 0.001	0.057
Propionate	245	232	230	232	237	4.8	0.148	0.263	0.028
Butyrate	106	116	119	129	130	2.7	0.002	< 0.001	0.125
Isobutyrate	10.6	11.2	10.9	12.2	12.5	0.47	0.011	< 0.001	0.504
Valerate	18.5	19.9	24.8	22.5	22.2	1.57	0.072	0.023	0.088
Isovalerate	13.3	14.4	14.5	14.5	16.4	0.85	0.116	0.017	0.568
pH	6.33	6.33	6.30	6.26	6.27	0.024	0.09	0.015	0.776
Net NH_3-N (mg/dL)	16.2	18.6	19.2	21.2	21.4	13.10	0.016	< 0.001	0.543

The forage-to-concentrate (F:C) ratios of experimental diets were 100:0 (100 F), 80:20 (80 F), 60:40 (60 F), 40:60 (40 F), and 20:80 (20 F).
SEM: highest standard error of mean. Lin: linear effect of increasing dietary inclusion of concentrate. Quad: quadratic effect of increasing dietary inclusion of concentrate.

higher proportion of CH_4 relative to TGP as fermentation progresses. This implies that microbial fermentation remained active throughout the incubation. In contrast, Serment et al. (2016) observed a slowing of fermentation rates toward the end of the incubation, attributing this to a decline in readily fermentable substrates and microbial activity.

4.2. Model adjustments and mean retention time considerations

In vitro models are valuable for screening dietary effects on fermentation parameters; however, they may not fully capture *in vivo* microbial interactions. There are several *in vivo* studies showing that higher levels of concentrates in the diet (above 40 %) alter CH_4 intensity (Hatew et al., 2015; Hristov, 2024). Furthermore, Cabezas-Garcia et al. (2017) found no difference in CH_4 production *in vivo* when gradually replacing highly digestible silage with low-digestibility silage and barley, and Agle et al. (2010) reported no variation in CH_4 production between diets with differing F:C ratios (48:52 vs. 28:72). This highlights the complexity of dietary effects on enteric CH_4 emissions, suggesting that factors such as rumen passage rate, microbial adaptation, and protozoal activity could influence CH_4 yields beyond what is captured *in vitro*.

Generally, increasing the concentrate proportion in the diet decreases CH_4 production (Hristov, 2024), whereas our results indicated the opposite. Therefore, we modified our prediction model to consider different ruminal MRT. We also included an extreme diet with a 20:80 F:C ratio, which is not relevant for practical feeding, but serves as a benchmark for *in vitro* model adjustments. The original *in vitro* study by Ramin and Huhtanen (2012) employed a modelling approach to predict *in vivo* CH_4 production using a fixed retention time of 50 h, assuming similar passage rates for forage and concentrate. This MRT was chosen to represent a cow fed at maintenance level.

More recently, Fant and Ramin (2024), predicted *in vivo* CH_4 production from an *in vitro* experiment and compared the results with CH_4 emissions measured *in vivo* by the GreenFeed system in lactating dairy cows. The same diets were used in both experiments. In addition to the 50 h MRT, a shorter 35 h MRT was added to the model to better reflect conditions during lactation, when higher feed intake leads to faster rumen passage rates. According to Krizsan et al. (2010), a 35 h MRT corresponds to a dry matter intake of 20 kg/day, closely matching the actual intake of 23 kg DM/day reported by Fant and Ramin (2024). In the study reported by Fant and Ramin (2024) using the 35 h MRT yielded CH_4 predictions that were not only lower than those based on 50 h, but also more closely aligned with the observed *in vivo* emissions. In fact, model fit improved substantially, with R^2 increasing from 0.56 to 0.91, and the root mean square error decreasing from 0.45 to 0.20. In the current study, decreasing MRT also led to reduced CH_4 production, supporting findings from previous studies (Goopy et al., 2014; Pinares-Patiño et al., 2003). This relationship is biologically plausible, as shorter MRT reduces the time available for fiber fermentation in the rumen, resulting in lower digestibility and, consequently, decreased CH_4 emissions, an effect noted as early as Blaxter and Clapperton (1965).

In addition, Huhtanen et al. (2015) showed that different retention times for forage and concentrate impact *in vivo* CH_4 predictions in mechanistic models, such as the Karoline model. The Karoline model is a dynamic, mechanistic model designed to simulate digestion, metabolism, and CH_4 production in dairy cows (Danfær et al., 2006; Huhtanen et al., 2015). It incorporates selective retention of feed particles, where forage particles remain in the rumen 1.6 times longer than concentrate particles (Cannas et al., 2003), influencing microbial fermentation dynamics and CH_4 output. In contrast to single-compartment models, Karoline describes the rumen as a two-compartment system in which feed particles must transition from a non-escapable pool to an escapable pool prior to passage, thereby more accurately representing the process of ruminal digestion. The improved representation of passage kinetics has been shown to enhance the accuracy of CH_4 predictions (Huhtanen et al., 2015; Ramin and Huhtanen, 2012).

We implemented the concept of specific MRTs for each evaluated F:C ratio following the approach used in the Karoline model. As CH_4 yield increased over time, integrating MRT adjustments reduced the differences in CH_4 production across different F:C ratios, without altering the statistical significance of the treatment effect. This was indicated by the smaller discrepancies between predicted CH_4 production values in the original and modified models. These adjustments emphasize the importance of passage rate and MRT in improving the accuracy of enteric CH_4 emission predictions based on *in vitro* data. However, *in vitro* models do not account for the

increased microbial efficiency associated with shorter retention times *in vivo*, which can influence CH_4 production estimates (Huhtanen et al., 2015).

4.3. Fermentation characteristics

Total VFA production was not affected by differing F:C ratios in this study, aligning with findings from an *in vitro* study by Serment et al. (2016), who noted no effect of diet on total VFA concentration. Instead, they reported that the inoculum affected the total VFA concentration, with higher total VFA being reported for the inoculum from donor cows adapted to high-concentrate diets (Serment et al., 2016). In contrast, Kim et al. (2018) and Vera et al. (2025) reported increased VFA concentrations in high-concentrate diets *in vitro*.

Most individual VFA proportions were linearly influenced by diet composition, except for propionate, which remained relatively stable despite increasing concentrate proportions. This result is somewhat unexpected, as higher starch proportions generally promote propionate production (Hristov, 2024; Kim et al., 2018). However, Jaakkola and Huhtanen (1993) reported that even with 75 % concentrate, propionate levels did not increase, with the lowest values observed at 50 % concentrate. This suggests that, in high quality grass silage-based diets, a very high grain inclusion may be required to significantly alter propionate levels. Starch-fermenting bacteria can compete with methanogens for H_2 utilization (Aguerre et al., 2011; Moss et al., 2000), which could influence fermentation patterns. Other studies have reported an increase in propionate with high-concentrate diets (Kim et al., 2018; Vera et al., 2025), but this response may depend on the forage source and the level of concentrate inclusion.

Acetate proportions were highest in the 100 F diet, which is expected for high-fiber diets, as acetate is primarily produced by cellulolytic microorganisms (Bačėninaitė et al., 2022; Jentsch et al., 2007; Wang et al., 2016). The inclusion of concentrate reduced acetate production, likely due to the inhibition of fibrolytic bacteria (Agle et al., 2010), leading to a decreased acetate to propionate (A:P) ratio. A lower A:P ratio generally indicates improved feed efficiency (Liu et al., 2019). While Kim et al. (2018) and Vera et al. (2025) also observed a decrease in A:P with higher concentrate levels, their studies reported a reduction in acetate alongside an increase in propionate. Nevertheless, our findings are consistent with previous studies that reported a decrease in A:P ratio with high-concentrate diets (Hristov et al., 2013; Jentsch et al., 2007).

Unlike acetate, butyrate proportion increased with concentrate inclusion in our study. Kim et al. (2018) and Vera et al. (2025) similarly observed higher butyrate levels in high-concentrate diets. While butyrate is often linked to protozoal metabolism *in vivo*, the higher proportions observed here are more likely explained by bacterial pathways favored under high-concentrate conditions. Starch-rich substrates support butyrate-producing bacteria such as *Butyrivibrio* spp. (Hua et al., 2017) and shifts in fermentation stoichiometry under buffered *in vitro* conditions may also direct reducing equivalents into butyrate rather than propionate.

Additionally, BCVFA, such as isobutyric and isovaleric acids, increased with higher concentrate levels. Branched chain VFAs are primarily produced during protein fermentation, and their higher concentrations indicate increased protein degradation and microbial synthesis in the rumen (Muller, 1987; Russell and Hespell, 1981). Since microbes typically incorporate amino acids into their own biomass rather than breaking them down into BCVFA, the increased BCVFA concentrations suggest reduced microbial biomass formation (Agle et al., 2010; Muller, 1987). The BCVFA play an essential role in microbial metabolism, as they are used in amino acid and fatty acid synthesis (Roman-Garcia et al., 2016) and have been shown to enhance fiber digestion (Muller, 1987). Roman-Garcia et al. (2016) reported that increasing BCVFA molar proportions improved microbial protein synthesis efficiency, particularly when NH_3-N levels also increased. However, when both NH_3-N and BCVFA levels were very high, microbial protein synthesis efficiency declined (Firkins et al., 2024). The observed changes in BCVFA and fermentation patterns in our study likely reflect shifts in microbial fermentation pathways rather than differences in protein supply.

In this study, NH_3-N concentrations increased linearly with increased concentrate inclusion, reaching the highest levels in the 20 F diet. All diets were balanced for CP content. During ruminal protein digestion, microbial proteolysis primarily produces NH_3-N, which serves as a crucial precursor for microbial protein synthesis (Putri et al., 2021). Efficient microbial protein synthesis is influenced by the synchronization of N and energy sources, with NH_3-N often playing a key role in this process (Agle et al., 2010). However, Khalili and Huhtanen (1991) suggested that NH_3-N may not always be limiting, particularly when alternative N sources, such as those from rapeseed meal, are available. The elevated NH_3-N concentrations observed here likely reflect increased protein degradation and deamination of rapeseed meal protein, along with a possible imbalance between the release of nitrogen and fermentable energy under the *in vitro* conditions (Zhang et al., 2020). Excess NH_3-N that is not incorporated into microbial protein is typically excreted in urine, leading to nutrient loss for the animal and increased environmental pollution (Agle et al., 2010). Previous studies have reported contrasting effects of concentrate inclusion on NH_3-N levels. While Arbabi et al. (2017) (*in vitro*) and Agle et al. (2010) (*in vivo*) observed a decline in NH_3-N with higher concentrate levels (average dietary CP of high concentrate diets was 28 % and 16.5 %, respectively), suggesting that fermentable carbohydrates may reduce deamination and enhance microbial ammonia utilization, Vera et al. (2025) found no significant effect of dietary concentrate on ruminal NH_3-N concentrations.

In our study, ruminal pH decreased with the inclusion of more than 40 % of concentrate but remained within the physiological range (5.5–7.5; Vadroňová et al. 2023), consistent with previous findings (Azmi et al., 2020; Serment et al., 2016; Vera et al., 2025). One critical factor influencing fermentation, the rumen microbiome, CH_4 production, and VFA concentrations is pH (Kim et al., 2018). A ruminal pH below 6.0 is known to suppress methanogenesis by inhibiting methanogenic activity (Arbaoui and De Vega, 2023; Serment et al., 2016). Lana et al. (1998) demonstrated that *in vivo* ruminal pH was strongly correlated with the capacity of bacteria to produce CH_4 *in vitro*, and that experimentally lowering *in vitro* pH from 6.5 to 5.7 reduced CH_4 production by approximately 85 %. These findings emphasize the close link between pH and methanogenesis and indicate that *in vitro* systems, because of their buffering capacity, may underestimate the effect of pH compared with *in vivo* conditions. Indeed, *in vitro* studies often maintain more stable pH

levels due to the presence of buffer systems in the inoculum (Serment et al., 2016). This was also the case in our experiment, where the mineral solution (Menke and Steingass, 1988) and the ratio between rumen fluid and mineral solution ratio (20:80 % v/v) likely mitigated the pH declines that would normally occur *in vivo*, particularly in high-concentrate, low-forage treatments. This stabilization may also lead to an overestimation of CH_4 production compared with *in vivo* scenarios where pH is lower (Danielsson et al., 2017). This limitation should be considered when extrapolating model predictions, and future validation under more variable ruminal pH conditions is warranted both *in vitro* and *in vivo*.

4.4. In vitro degradability

The OMD differed across diets and followed a quadratic pattern, where concentrate inclusion increased degradability from 100 F to 60 F, but then slightly decreased at 40 F and 20 F. The trend follows the same concept as described by Ramin and Huhtanen (2013) showing that digestibility and CH_4 production are positively correlated. The highest OMD was observed with the 60 F diet in our study, possibly because of relatively balanced diet, where the available fermentable material provided energy for the microbiota, enhanced their activity, and stimulated the digestion of forages (Getachew et al., 2005a). The higher degradability observed with the 60 F diet may be partially attributed to the ruminal fluid originating from cows fed a 60:40 diet. The forage in our study was highly digestible due to low iNDF content and had a high energy value. The concentrate in our study mainly consisted of rapidly fermentable barley grain (Hatew et al., 2015), and, generally, concentrates contain more digestible nutrients than forage, since concentrates have less cell wall components (Hristov, 2024). However, at very high concentrate levels (40 F and 20 F) in our study, concentrate may have negatively affected fiber degradability (Benchaar et al., 2001), which lead to the minor drop in OMD. Similarly, forage digestion was depressed with increased barley grain supplementation in Getachew et al. (2005a). This effect may be linked to a shift toward starch-utilizing microbes over fiber-degrading ones, and by causing local buildups of fermentation products that can hinder fiber digestion (Zhang et al., 2025). Excessive starch inclusion, therefore, did not further improve degradability, and instead may have shifted toward less efficient pathways that were less efficient at breaking down OM.

The relationship between concentrate and forage degradability is complex and influenced by factors such as intrinsic degradability and the level of supplementation. Published studies do not necessarily agree on the effects of concentrate supplementation on NDF degradability. Some claim that concentrate maximizes energy and protein utilization, others note no effect or even an increase in fiber degradability (Agle et al., 2010; Arbabi et al., 2017). Arbabi et al. (2017) reported a trend of increasing degradability with more concentrate, but a statistical significance was only observed for the 100 F diet compared with other treatments.

Despite the advantages of using an *in vitro* system such as the ability to screen large numbers of diets and study fermentation kinetics there are certain limitations. One key factor is that the rumen inoculum was not specifically adapted to high-concentrate diets, as the donor animals were fed a 60:40 F:C ratio. Studies suggest that incubating grains in rumen fluid adapted to the specific grain type can enhance fermentation efficiency, as the microbiota in adapted rumen fluid degrades starch-rich substrates more rapidly (Alvarez-Hess et al., 2019; Arbaoui and De Vega, 2023). As a result, the composition of the donor animal's diet can influence TGP (Nagadi et al., 2000). Further limitation relates to the buffer system used *in vitro*, which keeps pH relatively stable. This can be particularly important when evaluating high-concentrate diets, as rumen pH would normally decline under such conditions, suppressing methanogenesis (Lana et al., 1998; Muetzel et al., 2014). By preventing this pH drop, the *in vitro* system may lead to an overestimation of CH_4 production from concentrate-rich diets. In contrast, this effect is less critical for forage-based diets, where rumen pH tends to remain more stable even *in vivo*.

Another inherent limitation of *in vitro* studies is their closed nature with no passage of feed particles, which may not fully replicate *in vivo* fermentation dynamics. However, incorporating modelling approaches helps mitigate these discrepancies, producing more reliable results and reinforcing the value of *in vitro* systems as a tool for dietary screening (Chagas et al., 2019; Danielsson et al., 2017).

5. Conclusion

This study presents a refined *in vitro* model that incorporates MRT to better simulate rumen fermentation dynamics and enteric CH_4 emissions across different F:C ratios. Adjusting for MRT may enhance CH_4 prediction accuracy and better reflect shifts in degradability and fermentation profiles, as indicated by comparisons with previous studies, especially at intermediate F:C ratios. These findings emphasize the importance of accounting for passage kinetics in *in vitro* systems to improve their applicability to *in vivo* conditions and enable more reliable evaluation of dietary strategies for enteric CH_4 mitigation. Importantly, this model represents a preliminary step rather than a final validation, and further work is required to test its performance against *in vivo* data using wider range of practical diets.

CRediT authorship contribution statement

Mohammad Ramin: Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Mariana Vadroňová:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Petra Fant:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Giorgio Menni:** Writing – review & editing, Software, Formal analysis.

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

This research was financed by Stiftelsen Seydlitz MP bolagen (27455000), and carried out using data and materials from Röbäcksdalen, SITES (Swedish Infrastructure for Ecosystem Science), a national coordinated infrastructure funded by the Swedish Research Council.

Data availability

None of the data were deposited in an official repository. The data will be made available upon reasonable request.

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