Different forage-to-concentrate ratios in the diet of dairy cows and predicted in vivo methane production.

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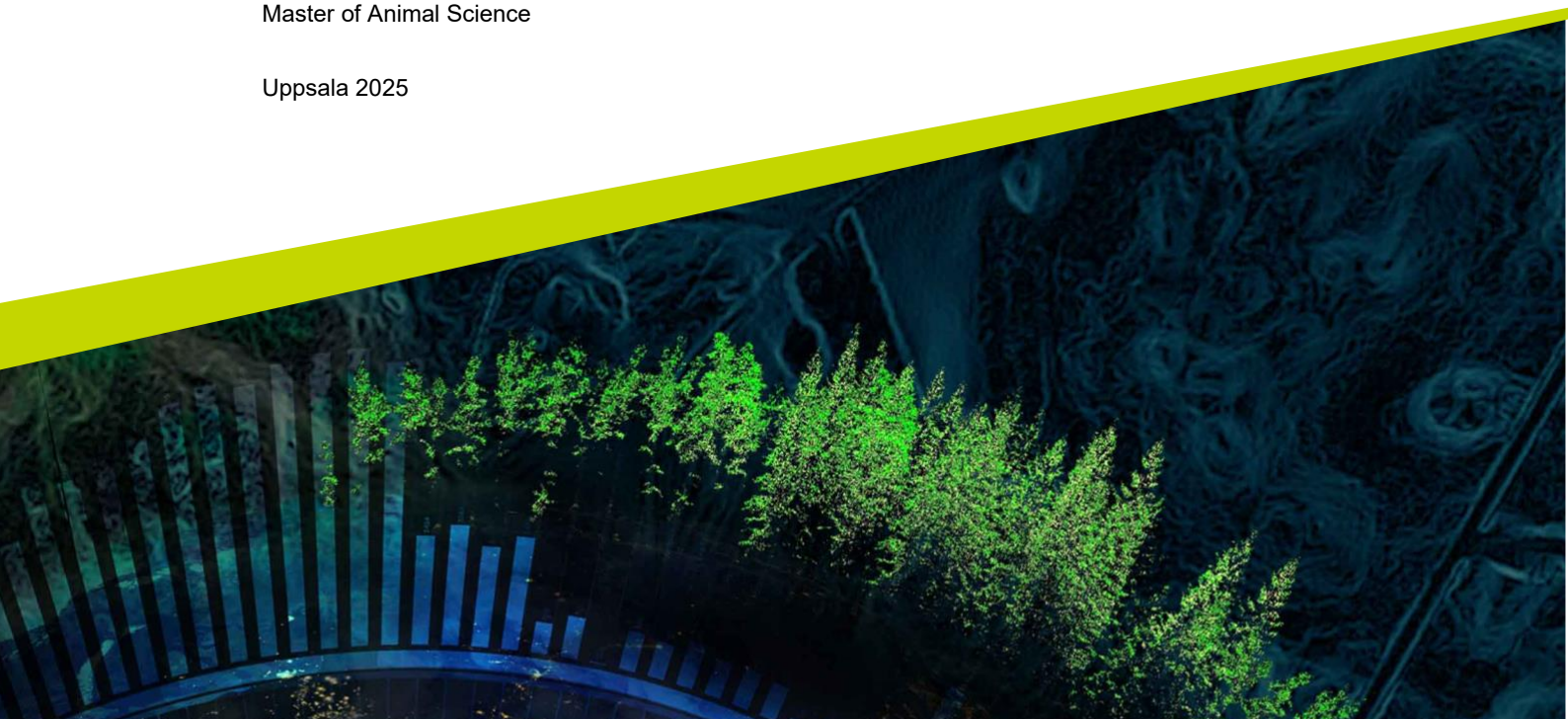

Different forage to concentrate ratios in the diet of dairy cows and predicted in vivo methane prediction.

Olika grovfoder- till kraftfoderförhållanden i foderstaten till mjölkkor och skattade in vivo metanutsläpp

Luis Alberto Duarte Gonzalez

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Abstract

As the global food demand continues to rise and projected to reach 9.7 billion by 2050. The adoption of sustainable livestock practices becomes vital, particularly in regions like the Nordic countries that are heavily dependent on dairy production. Ruminants play a crucial role in converting fibrous plant material into high-quality protein, but they also contribute significantly to methane (CH_4) emissions, a potent greenhouse gas and an energy loss source in cattle.

This research explored the impact of varying forage-to-concentrate (F:C) ratios by using in vitro methodology to estimate total gas and CH_4 production from diets containing grass silage as the forage source and a concentrate mixture of barley and rapeseed meal. The experiment involved two incubation runs with five dietary treatments (100:0, 80:20, 60:40, 40:60 and 20:80) and four replicates per treatment. Rumen fluid was collected from two cannulated dairy cows and incubated with feed samples in anaerobic serum bottles. The samples were incubated at 39°C, with automatic gas production measurements taken every 12 minutes. Methane concentration was analyzed at 2, 4, 8, 24, and 48 hours using gas chromatography. The pH and volatile fatty acids (VFA) were measured at the end of the incubation from each replicate. To determine total dry matter digestibility (TDMD) and total organic matter digestibility (TOMD), samples were randomly selected. Data were analyzed using a MIXED procedure of SAS® 9.4, with treatment and run as fixed effects and bottle as a random effect. The results indicated that increasing concentrate levels led to a significant linear rise in total gas ($P < 0.001$), and CH_4 production ($P < 0.001$), along with a tendency for a decrease in pH ($P = 0.09$). Acetic acid concentrations decreased linearly ($P < 0.001$), while butyric acid increased ($P = 0.002$), indicating a shift in fermentation patterns. Although total VFA concentrations and propionate proportion remained stable. TDMD and TOMD peaked at intermediate F:C levels (60F and 40F), suggesting optimal nutrient utilization at these ratios. In addition, the observed increase in CH_4 production at higher concentrate levels may be partially attributed to the nature of the in vitro system itself. Unlike the rumen environment in vivo, where there is continuous absorption of end products and passage of feed particles, the in vitro batch fermentation setup functions as a closed system.

These findings suggest that diet composition significantly influences fermentation dynamics and CH_4 output, underscoring the potential for manipulating F:C ratios to enhance feed efficiency and reducing CH_4 emissions in ruminant systems. However, the limitations of in vitro models, including the lack of absorption and microbial adaptation, require cautious extrapolation to in vivo scenarios.

Keywords: Methane, Ruminants, Methane mitigation, In vitro gas production.

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Abbreviations

Abbreviation	Description
CH_4	Methane
DMD	Dry Matter Digestibility
F:C	Forage to Concentrate
VFA	Volatile Fatty Acid
TDMD	Total Digestible Dry Matter
BRF	Buffer Rumen Fluid
RSM	Rapeseed meal
RF	Rumen Fluid
NH_3-N	Ammonia nitrogen
SLU	Swedish University of Agricultural Sciences

1. Introduction

1.1 Sustainable Practices in Nordic Dairy Farming

The projected world population by 2050 is 9.7 billion people (United Nations 2015). As the global population continues to grow, the demand for food is expected to increase significantly. Meeting this rising demand presents major challenges for global food security and underscores the need to scale up food production in a way that is both economically viable and environmentally sustainable (Berners-Lee et al., 2018). In addition, the global cattle population has increased significantly in response to rising demand for meat and dairy products, particularly in developing countries (Ahmad 2001; Berners-Lee et al. 2018). This expansion presents a major challenge for producers of high-yielding dairy cattle: As the livestock systems intensify, especially those focusing on high-output breeds, optimizing feed composition becomes essential.

Animal feed is one of the largest operational costs for livestock producers improving feed efficiency particularly through combinations of hig-quality forage, cereal grains, and protein sources such as rapeseed meal- is key to both economic viability and long-term food security. (Dijkstra et al. 2011; Hammond et al. 2015). Cattle and other ruminants, such as sheep and goats, have a unique digestive system which allow them to break down fiber that monogastric animals and humans cannot digest. This process enables ruminants to utilize various agricultural byproducts (Oltjen & Beckett 1996; Dijkstra et al. 2011). Nevertheless, the microbial fermentations that occur in the rumen produces CH_4 as a byproduct, and as the global ruminant population grows, the amount of CH_4 released into the environment increases. Livestock production is estimated to cause approximately 14 to 18 percent of the world's anthropogenic greenhouse gas emissions (Beauchemin et al. 2007; Tedeschi et al. 2022).

In Nordic countries, dairy farming serves as a fundamental pillar of the agricultural sector. This is characterized by the utilization of high-yielding dairy breeds and well-implemented feeding system (Zira et al. 2025). The primary feed sources for dairy cattle in this region consist of high-quality forages, including grass and legume silage (Spörndly & Nilsdotter-Linde 2011). These forages are supplemented with concentrates such as barley and rapeseed meal to optimize milk production (Sairanen et al. 2022). Due to the short growing season and cold climate, Nordic dairy farmers prioritize feed preservation techniques, such as silage production to maintain a consistent year-round supply of nutrients (Sairanen et al. 2022).

The policies of Nordic countries strive to reduce CH_4 emissions by enforcing strict environmental and sustainability regulations. These policies encourage efficient resource utilization in the dairy production (Sairanen et al. 2022). Consequently, practices such as improved forage management and higher milk yields have contributed to reduced emission intensity. Although genetic selection for more

feed-efficient animals—such as through the recent inclusion of residual feed intake (RFI) in breeding goals—holds promise for future reductions, its effects on methane emissions have not yet been realized in Nordic dairy herds(Beauchemin et al. 2020). National and EU regulations endorse sustainable livestock production by motivating CH_4 reduction strategies. These strategies include optimizing feeding management and incorporating dietary additives, such as tannins and essential oils, to modify rumen fermentation (Sairanen et al. 2022).

In addition to environmental concerns, economic factors play an essential role in shaping Nordic dairy farming practices. Due to the high costs associated with purchasing feed, farmers are increasingly turning to locally sourced alternatives (Zira et al. 2025). This shift has prompted both farmers and researchers to explore improved feeding strategies aimed at reducing diet cost while maintaining or improving productivity and sustainability. Recent studies have shown that altering the forage-to-concentrate ratio (F:C) can significantly enhance feed efficiency and reduce CH_4 emissions particularly reducing the proportion of low-digestibility forage or increasing the inclusion of high-quality forage can improve feed efficiency and reduce enteric CH_4 emission in ruminants (Min et al. 2022). Adjusting, the F:C ratio in the diet presents a feasible strategy that can benefit both the environment and the profitability of the farmers.

Therefore, finding strategies to mitigate CH_4 production in ruminants is crucial to improving both environmental sustainability and animal productivity. Various strategies have been explored, such as dietary modifications, feed additives, and microbial interventions, to reduce CH_4 emission without compromising animal health or performances. Research in this area continues to evolve, with promising results indicating that it is possible to address this issue effectively while still maintaining an efficient livestock production system.

1.2 The Rumen Microbial Physiology and Methanogenesis

The rumen, a complex fermentation chamber, plays a crucial role in ruminants by breaking down and digesting feed (Figure 1). It hosts a diverse and dynamic microbial community that contributes significantly to CH_4 production through the process of methanogenesis (Waters et al. 2025). This process, which is primarily carried out by methanogenic archaea, depends on a limited range of substrates, particularly hydrogen (H_2), carbon dioxide (CO_2), formate, acetate, and alcohols (Hook et al. 2010; Zabranska & Pokorna 2018; Almeida et al. 2021). These substrates are primarily obtained during the fermentation of carbohydrates, proteins, and lipids by fermentative bacteria in the rumen (Dijkstra et al. 2011; Olijhoek et al. 2016).

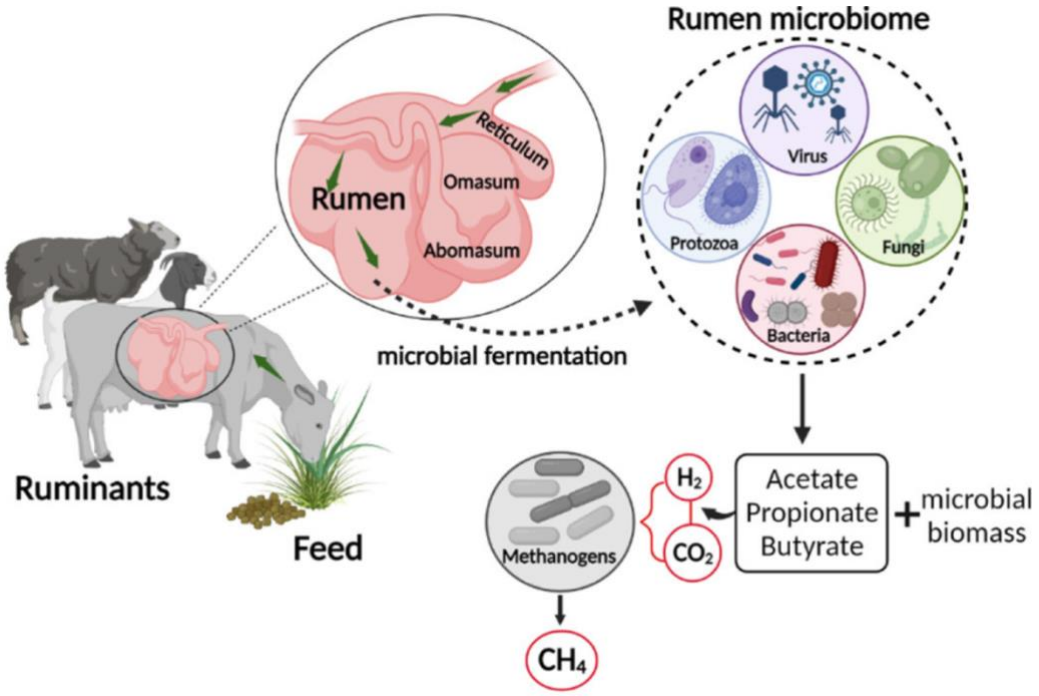

Figure 1. An overview of rumen morphology and microbiome by Smith et al. (2024)

The microbial ecosystem in the rumen comprises bacteria, archaea, protozoa, fungi, and viruses. Each of these microorganisms plays a distinct role in the fermentation and breakdown of organic material. Methanogens, are the primary consumers of H_2, maintaining a low partial pressure of hydrogen within the rumen environment (Moss et al. 2000; Zabranska & Pokorna 2018). The condition favours methanogenesis as the dominant hydrogen-consuming pathway. Additionally, preventing other hydrogen-utilizing microbes, such as acetogens and sulphate-reducing bacteria, from outcompeting methanogens for this crucial substrate (Olijhoek et al. 2016; Zabranska & Pokorna 2018).

The pH of the rumen significantly influences microbial activity and fermentation patterns. It typically ranges between 5.5 and 7.0, depending on diet composition (Soest 1994; Council et al. 2001). High-forage diets tend to maintain a more neutral pH, whereas high-grain diets, rich in fermentable carbohydrates, result in a more acidic environment (Soest 1994). The pH shift significantly affects microbial populations and fermentation products. For example, a decrease in rumen pH, often associated with high-grain diets, promotes the growth of propionate-producing bacteria, which compete with methanogens for hydrogen utilization (Morgavi et al. 2012; Vyas et al. 2014; Beauchemin et al. 2020). The production of propionate is an alternative hydrogen sink, reducing CH_4 emissions by utilizing hydrogen (Vyas et al. 2014; Olijhoek et al. 2016). This shift in fermentation patterns from acetate and butyrate toward propionate results in lower CH_4 production. This change reflects the microbial competition for hydrogen (Ellis et al. 2008). Methanogens, which are sensitive to low pH, decrease under these conditions, as acidic environments inhibit their metabolic processes (Hristov et al. 2013; Zabranska & Pokorna 2018).

Protozoa also play a vital role in the rumen microbial ecosystem. These single-cell organisms consume bacteria and other microbes, which influence the overall fermentation process. Protozoa produce H_2 and CO_2 as byproducts of fermentation, which are then directly transferred to methanogens (Morgavi et al. 2012). This symbiotic relationship helps to maintain low hydrogen partial pressures and promoting methanogenesis (Morgavi et al. 2012; Zabranska & Pokorna 2018). However, the removal of protozoa, or "defaunation," can disrupt microbial interactions and lead to a decrease in CH_4 production (Ellis et al. 2008; Morgavi et al. 2012; Zabranska & Pokorna 2018). Protozoa defaunation alters fermentation profiles, retention time, and nutrient absorption, which may further impact overall rumen function (Lovett et al. 2003; Ellis et al. 2008).

Understanding the microbial competition for hydrogen in the rumen, along with interactions between methanogens, propionate producers, sulphate-reducing bacteria, and protozoa, is essential for developing effective strategies to mitigate CH_4 emission from cattle. Rumen physiology, particularly pH and substrate availability, directly influences these microbial interactions and fermentation dynamics.

1.3 Strategies to Lower Methane Emissions

Various dietary interventions have been explored to mitigate CH_4 emissions while maintaining ruminant productivity. These strategies include modifications in forage quality, feed additives, lipid supplementation, and plant secondary metabolites, among others (Danielsson et al. 2017; Chagas et al. 2019).

The F:C ratio in dairy diets significantly influences rumen microbial activity and CH_4 emissions. Microbial fermentation, driven by bacteria, yeast, and fungi, plays a key role in converting feed into high-quality microbial protein (Min et al. 2022). However, this microbial fermentation produce CH_4, which reduce the animal`s efficiency in energy utilization due to lose of energy via eructation into the environment (Johnson & Johnson 1995; Ramin & Huhtanen 2012; Bell et al. 2016). This energy loss not only has negative environmental impacts but also compromise the efficiency of the feed conversion in ruminants, thus affecting overall animal productivity (Caro et al. 2014; Moraes et al. 2014; Min et al. 2022).

The type and quality of forage significantly influences CH_4 emissions. High-quality forages with increased digestibility reduce CH_4 production by promoting rapid fermentation and altering rumen fermentation pathways (Dittmann et al. 2016; van Gastelen et al. 2019). Legume-based forages, such as clover and alfalfa, contain less fiber and higher protein content than grasses. They have been shown to decrease CH_4 emissions primarily because they contain less fiber, resulting in reduced fermentation and, consequently, lower methane production by the rumen microbes (Olijhoek et al., 2016; Grant & Ferraretto, 2018; van Gastelen et al., 2019).

Increasing the concentrate proportion in the diet enhances starch fermentation and promotes propionate production, which serves as an alternative hydrogen sink, reducing CH_4 emission (Aguerre et al. 2011; Olijhoek et al. 2018).

Diets high in rapidly fermentable starches, such as those containing large amounts of oats or corn, can help reduce CH_4 emissions. However, excessive starch fermentation in the rumen can lead to a rapid drop in pH, increasing the risk of ruminal acidosis and disrupting the microbial balance (Penner et al., 2009). Finding an optimal concentrate proportion that minimizes CH_4 emissions while maintaining rumen health is crucial for sustainable ruminant nutrition.

Moreover, lipid supplementation has emerged as a promising mitigation strategy. Adding fats, such as coconut oil, linseed oil, or soybean oil, to ruminants' diets reduces enteric CH_4 emissions by directly inhibiting methanogenic archaea and decreasing fiber fermentation (Lovett et al. 2003). A study showed that coconut oil supplementation reduces DMI and gross energy intake while maintaining milk production, leading to a lower CH_4 emissions and a decrease in ruminal protozoa population (Lovett et al. 2003). However, excessive lipid inclusion (>6% of total dry matter) can negatively affect fiber digestibility by coating feed particles and inhibiting the activity of fiber-degrading microbes in the rumen. This microbial suppression can also lead to reduced feed intake, highlighting the importance of controlled lipid supplementation (Beauchemin et al., 2020).

In addition to lipid supplementation, the use of grain type sources, such as replacing traditional grains with fibrous by-products, can also influence CH_4 emission. For instance, Ramin et al. (2021) reported that replacing barley with oats in dairy cow diets reduced CH_4 emissions by 4.6% to 4.8% without affecting milk yield, making it a viable option in oat-producing regions.

Another strategy to reduce CH_4 emissions involves the use of feed additives such as certain types of red algae, particularly Asparagopsis species. These algae contain bromoform ($CHBr_3$), a bioactive compound that inhibits methanogenesis by disrupting the enzymes used by methanogenic archaea in the rumen. This suppression of methane-producing microbes can significantly reduce CH_4 emissions from ruminants (Chagas et al. 2019). For example, an in vitro study found that adding *Asparagopsis taxiformis* to the diets of cattle reduce CH_4 emission by up to 79% (Machado et al. 2016). This highlights the potential of utilizing bioactive compounds from *Asparagopsis taxiformis* as a sustainable solution to mitigate greenhouse gas emission from livestock production.

1.4 Techniques to Measure CH_4 emission

Several methods of CH_4 measurement have been developed to quantify CH_4 production and emission from ruminants (Figure 2).

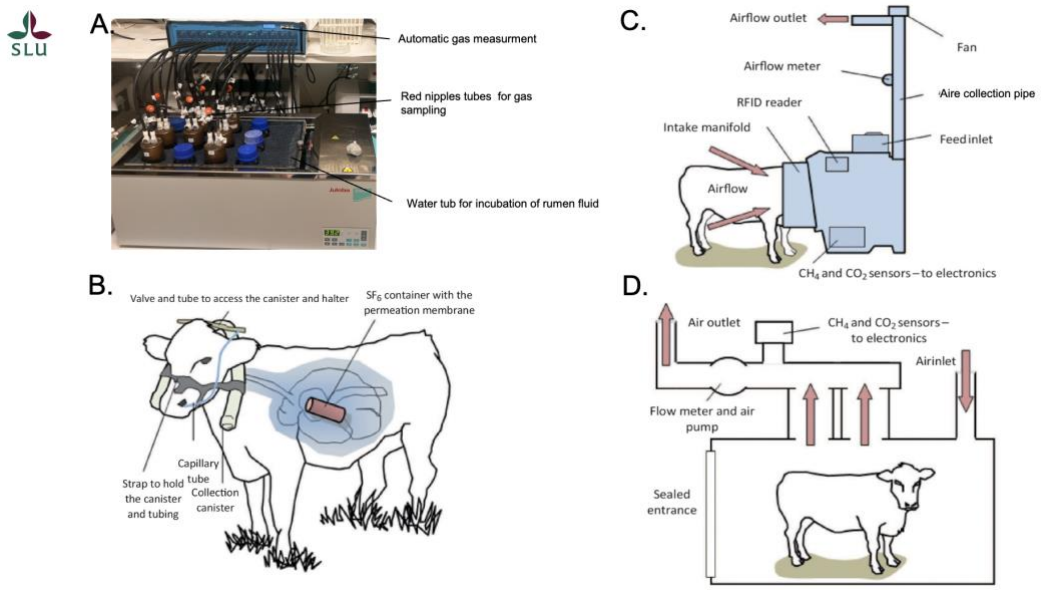

Figure 2. A comprehensive overview outlining various methodologies employed for CH_4 measurement is provided. Method A represents an in vitro approach for automatic gas measurement. Method B utilizes the sulphur hexafluoride (SF_6) tracer system. Method C encompasses the Greenfeed method, which is implemented through the C-lock software system. Method D involves respiration chambers modified from Hill et al., (2016).

Techniques applied directly to animals—such as the sulfur hexafluoride (SF_6) tracer method, GreenFeed systems, and respiration chambers—are considered highly accurate for measuring methane (CH_4) emissions and assessing nutrient use under practical conditions (Danielsson et al., 2017; Ma et al., 2024). These in vivo methods are recognized for their precision, with validation studies confirming the reliability of both GreenFeed and respiration chambers for CH_4 quantification (McGinn et al., 2021). However, Ma et al. (2024) report discrepancies in carbon dioxide (CO_2) and oxygen (O_2) measurements between the two systems, highlighting the need for caution when comparing results across methodologies. Despite their accuracy, these approaches require significant resources, including specialized infrastructure, trained personnel, and strict adherence to ethical guidelines, which limits their practicality for large-scale studies (Huhtanen et al., 2015). Moreover, environmental factors and individual animal variability can affect measurements, often necessitating larger sample sizes to ensure robust conclusions (Ma et al., 2024).

In vivo methods are constrained by high costs, logistical challenges, and ethical considerations (Huhtanen et al. 2015; McGinn et al. 2021; Ma et al. 2024). Therefore, in vitro gas production (GP) methods, particularly those using automated gas measurement systems, are simpler and cost effective alternative methods (Ramin & Huhtanen 2012). Recently, the in vitro method has been employed to investigate dietary strategies and additives aimed at reducing CH_4 emissions from ruminants. These approaches offer significant advantages for researchers with limited resources, often serving as the primary tool for identifying promising agents for CH_4 mitigation.

The in vitro technique simulates rumen fermentation under controlled laboratory conditions by incubating feed samples with rumen fluid and buffer solutions. This setup enables the assessment of fermentation kinetics, gas production (including methane), volatile fatty acids, and ammonia concentrations. It offers a cost-effective, reproducible, and ethically favorable alternative to in vivo methods, allowing researchers to precisely control experimental conditions and test multiple treatments efficiently (Menke & Steingass 1988; Theodorou et al. 1994; Getachew et al. 2004).

In vitro, which involve incubating rumen fluid, has been widely used to assess the nutritional value of ruminant feeds (Yáñez-Ruiz et al. 2016). These methods, complement with the chemical analyses, offers a faster and less expensive alternative to in vivo methods. In vitro methods also minimizes the dependence on animal testing, particularly when evaluating multiple feed treatments (Ramin & Huhtanen 2012; Yáñez-Ruiz et al. 2016).

1.5 Study Purpose

The aim of this study is to evaluate predicted in vivo CH_4 production using an automated in vitro gas production system, building on the methodology established by Ramin and Huhtanen (2012). Moreover, it will demonstrate how different F:C ratios affect total gas and CH_4 emission. This study hypothesizes that varying F:C ratios in dairy cows' diets influence CH_4 production, as changes in diet composition alter the energy density and fermentation dynamics in the rumen.

2. Material and Methods

2.1 Experimental Design

The experimental design aimed to assess different levels of forage-to-concentrate (F:C) ratios utilizing grass silage as forage, barley and rapeseed meal (RSM) as concentrate components in diets. The experiment was conducted over two in vitro incubation runs.

In each run, a total of 26 bottles were incubated, with four replicates per treatments. The treatment consisted of varying F:C ratios: 100:0, 80:20, 60:40, 40:60, and 20:80. Additionally, six bottles were assigned as blanks, containing only buffered rumen fluid. As illustrated in Table 2, the proportion of barley and RSM decreased while the forage content increased (ranging from 20% to 100%). Each diet was designed to contain 20% crude protein on a dry matter (DM) basis. The percentage of ether extract decreases (from 3.8% to 2.3% DM) as the concentrate content of the diet increases.

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 silage1	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. Grass silage characteristics: NH3-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.

Table 2. Composition of Feed Ingredient in Different Treatments.

Treatment	Avg. Forage (g)	Avg. Barley (g)	Avg. RSM (g)	Avg. Weight (g)	CP	EE	Starch	NDF	iNDF
100F	1.002	-	-	1.0012	203	37.5	8.13	420	29.8
80F	0.8006	0.1209	0.0803	1.0018	203	34.0	81.9	380	37.0
60F	0.6004	0.2403	0.1605	1.0012	204	30.4	156	340	44.2
40F	0.4004	0.3609	0.2405	1.0018	204	26.9	229	300	51.5
20F	0.2014	0.4806	0.3206	1.0026	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 (100F), 80:20 (80F), 60:40 (60F), 40:60 (40F), and 20:80 (20F).

Samples for VFA and NH_3-N analysis were collected from two replicates of each treatment at the end of the incubation period. Using a syringe, 0.2 mL of the incubation fluid were poured into Eppendorf tubes and stored at -20 for subsequent analysis. The NH3-N level was quantified using a continuous flow instrument (AutoAnalyzer 3 HR; SEAL analytical, Southampton, UK) by measuring light absorption at a wavelength of 660 nm. Prior to analysis, the samples were thawed and centrifugated at 12,500 x g for 10 minutes. Subsequently, a 0.1 mL fraction of the clear upper layer was diluted using ultrapure water using a dilution rate of 1:25. The experimental process is illustrated in Figure 1, starting with the collection of rumen fluid from two cannulated cows, which is then filtered and mixed with feed samples in anaerobic 250 mL serum bottles. The mixture is incubated in a water bath to facilitate microbial activity, and gas production is measured using specialized software. Methane concentrations are analyzed using a gas chromatograph (Trace 1300 Gas Chromatograph; thermos scientific). Nylon bags with a pore size of 11 μm was used for filtering undigested feed particles for dry matter and organic matter digestibility after 48 hours (Rodrigues et al. 2018).

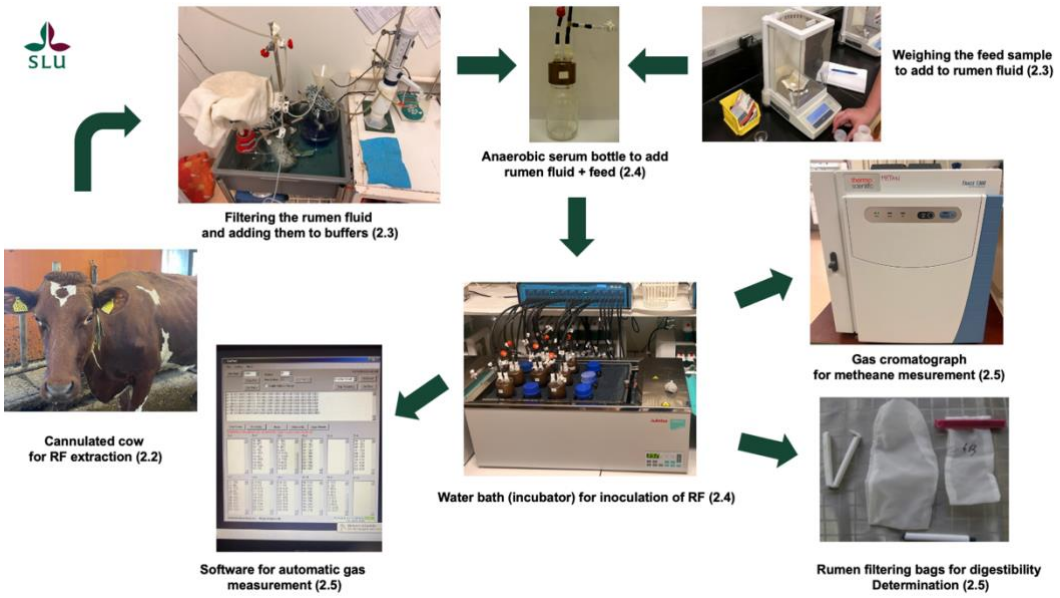

Figure 3. Experimental design for in vitro automatic gas production measurement and CH_4 production. Illustration created by Luis Duarte using PowerPoint.

2.2 Rumen Fluid Collection

The study consisted of a two-phase experiment conducted at the laboratory of Swedish University of Agricultural Science (SLU) in Umeå. The Swedish Ethics Committee on Animal Research approved the animal handling procedures (Dnr A 6-2021, and the study adhered to Swedish regulations for live animal experimentation. Rumen fluid was collected from two dairy cows in early lactation at SLU facility at Röbäcksdalen experimental farm in Umeå. The cows were fed ad libitum on a diet with a F:C ratio of 60:40 on a dry matter (DM).

The rumen fluid was collected through an open cannula and stored in a pre-warmed thermos flask at 39°C that had been flushed with CO_2 to preserve microbial activity. The rumen fluid was delivered to the SLU Umeå laboratory within approximately 15 minutes.

2.3 Feed Sample and Buffer Rumen Fluids Preparation

The preparation of the in vitro consisted in drying the feed samples at 60 °C for 48 hours to remove moisture and ensure uniformity. Once it dried, the feeds were grounded using a 1 mm screen to increase surface area for the microbial fermentation. A precise amount of forage, barley, and rapeseed meal was weighed. Approximately 1003 ± 30 mg of feed was carefully measured using an analytical balance and placed into a serum bottle.

The experiment was conducted using incubation bottles with airtight caps, a glass flask for buffer preparation, and a beaker for transferring rumen fluid. Funnels with four layers of folded cheesecloth were used for filtering the rumen fluid. The

chemical reagents included peptone (2 g), sodium sulphite (Na_2S) (475 mg), sodium hydroxide (NaOH) (3.3 ml), and resazurin (2.47 ml) for buffer preparation (Menke & Steingass 1988; Ramin & Huhtanen 2012). Additionally, deionized water (964.30 ml) and rumen fluid (483.12 ml) were used to maintain the appropriate condition. The CO_2 gas was used to establish anaerobic conditions.

Key equipment included a calibrated pH meter an auto-pipette (30 ml), and an analytical balance for sample weighing. Moreover, a shaking water bath at 39 °C and 40 rpm holding the bottle connected to the system for measuring gas production.

2.4 In Vitro Incubation

The water bath was filled with distilled water (without the bottles) to ensure that the production bottles were submerged to an adequate level. To prevent microbial contamination, 30 ml of an anti-bacterial growth/algae solution was added to the production baths. After preparing the incubation bottles, each bottle was pre-flushed with CO_2 before adding 60 ml of buffered rumen fluid (BRF). The bottles were tightly sealed, flushed again with CO_2 to create headspace, and connected to the gas monitoring system. The bottles metal head cap was made with a pair of pressure tubes linked to the gas monitoring. A three ways metal valve was attached to one of these pressure tubes with a rubber suba seal septa (Z124567–100EA, 13, Merck) placed in the third port for sampling and measuring CH_4. The samples were collected with a gas tight syringe from the suba seal septa connected to the tube. In the second pressure tube, a plastic tube was inserted to collect liquid samples which create a T-shaped tube equipped with a valve for liquid phase sampling (Figure 4). All bottles were incubated in a 39 °C water bath and covered with foam pieces to minimize temperature fluctuations.

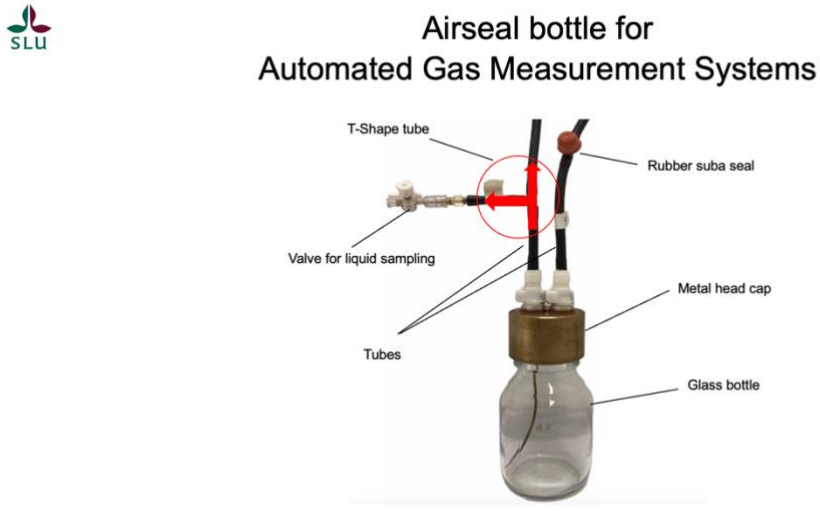

Figure 4. Airseal bottle for invitro incubation and automatic measurement of gas production. The red rubber suba seal that is in the tube serves as point to collect gas for CH4 measurements utilizing a gastight syringe. Illustration modified from Ramin et al., (2012).

2.5 Automatic Gas and CH_4 Measurement Sampling

Total gas production (CO_2, CH_4 and H_2) was recorded every 12 minutes using an automated system and corrected to standard air pressure described by Ramin and Huhtanen (2012). Methane measurements followed the protocol by Ramin and Huhtanen (2012), with samples of 0.2 mL were taken using a gas tight syringe from each bottle at 2, 4, 8, 24 and 48 hours. Methane concentration was measured with a Trace 1300 Gas Chromatograph (Thermos scientific, Italy).

At the end of the 48-hour incubation period, the gas production system was stopped, the water bath was turned off, the bottles were opened, and pH measurements were taken from all four replicates of each sample. Immediately after, two replicates of each treatment specifically designed for VFA analysis were collected.

For total dry matter digestibility (TDMD) and total organic matter digestibility (TOMD) analysis, the remaining two replicates for each treatment were placed on ice to stop any further microbial activity before washing and freezing. Then, the feed sample from each bottle were placed into nylon bags with an 11 μm pore size, following the method outlined by Rodrigues et al. (2018). Each bottle was scraped with a metal spatula to remove all the feed stuck to the glass wall. Liquid and feed particles were then poured into the nylon bags using a funnel to prevent particle loss. Moreover, the bottles were rinsed with a small volume of distilled water and the contents were funneled into the nylon bags. The bags were securely fastened with straps and then submerged in a neutral detergent solution. They were boiled for an hour with heat-stable α-amylase and sodium sulphites and neutral detergent solution to eliminate any microbial material holding to the feed residues. Afterward, the bags were rinsed thoroughly and then boiled in water for 10 minutes. Subsequently, they were dried in an oven at 60°C for 48 hours. Finally, the bags were weighed to determine the TDMD. To calculate TOMD, the residues were placed in crucibles and incinerated at 500°C for four hours.

Predicted CH_4 in vivo was calculated as described by Ramin and Huhtanen (2012). Predictions were made based on CH_4 concentrations measured at different time points, total gas as follow: Total methane production (ml) = headspace (HS) volume (ml) ×HS methane concentration + gas production (ml) × A × HS methane concentration.

The headspace volume in the system is 265 ml (the volume is for bottles and pressure tubes connected to the gas reader box). The total gas volume is automatically recorded by the system and corrected for the normal air pressure. Coefficient A is the ratio of methane concentration in outflow gas to HS (0.55) Ramin and Huhtanen (2012).

2.6 Statistical Analysis

The data from the experiments were analyzed using the MIXED procedure of SAS® 9.4 (SAS Institute Inc., 2025) using 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 (20:80; 40:60; 60:40; 80:20; and 100:00)
R_j= run effect: j = 2
B_k= bottle effect (K= 30)
e_{ijk}= residual error

T_i and R_j were considered fixed effects while B_k was treated as random effect. To evaluate the effect of different F:C ratios, linear and quadratic contrasts were performed. Differences between treatments were assessed as significant if $P \leq 0.05$, whereas a tendency toward significant was considered if $0.05 < P \leq 0.1$.

3. Results

The F:C ratio had a significant impact on total gas and CH_4 production ($P < 0.001$), (Figures 5 and 6). The pH tended to slightly decrease across treatments ($P = 0.09$) ranging from 6.33 in the higher forage treatment (100F) to 6.26 in the higher concentrate treatments (20F), indicating a decline with increase concentrate levels (Table 3).

The predicted total gas production (ml/g DM) showed an increasing linear trend ($P < 0.001$), rising from 243 at the highest forage treatment (100F), 256 (80F), 268 (60F), 287 (40F), and 295 at the lowest treatment (20F). Similarly, predicted CH_4 production (ml/g DM) increased from 37.7 (100F), 43.4 (80F),45.9 (60F), 48.4 (40F), and 49.0 (20F). Predicted CH_4 production showed both a linear ($P < 0.001$) and a quadratic ($P = 0.027$) relation.

Table 3. Gas Production and digestibility parameters of the different Forage-to-Concentrate Ratios.

Variable	100F	80F	60F	40F	20F	SE	P- Values T	L	Q
pH	6.33	6.33	6.33	6.26	6.27	0.024	0.09	0.015	0.776
Predicted total gas production (ml/g DM)	243	256	268	287	295	4.5	<0.001	<0.001	0.836
Predicted CH_4 production (ml/g DM)	37.7	43.4	45.9	48.4	49	1.19	<0.001	<0.001	0.027
TDMD%	88.9	90.0	90.4	90.4	89.8	0.364	0.009	0.023	0.003
TOMD%	89.3	90.7	91.4	91.2	90.4	0.421	0.01	0.006	<0.001
Total VFA mmol/g DM	6.07	6.58	6.05	6.32	6.47	0.27	0.492	0.468	0.909
Acetic acid mmol/l	606	604	595	590	578	2.23	<0.001	<0.001	0.057

Propionic acid mmol/l	245	232	230	232	237	4.87	0.148	0.263	0.028
Butyric acid mmol/l	106	116	119	129	130	2.65	0.002	<0.001	0.125
Net NH_3-N (mg/dL)	16.2	18.6	19.2	21.2	21.4	13.1	0.016	<0.001	0.543

The NH_3-N concentration shows a noticeable increase as higher concentrate inclusion is implemented in the diet. It begins at 16.2 mg/dL with the 100F diet. As the diet shifts to the 20F diet, the concentration reaches 21.4 mg/dL.

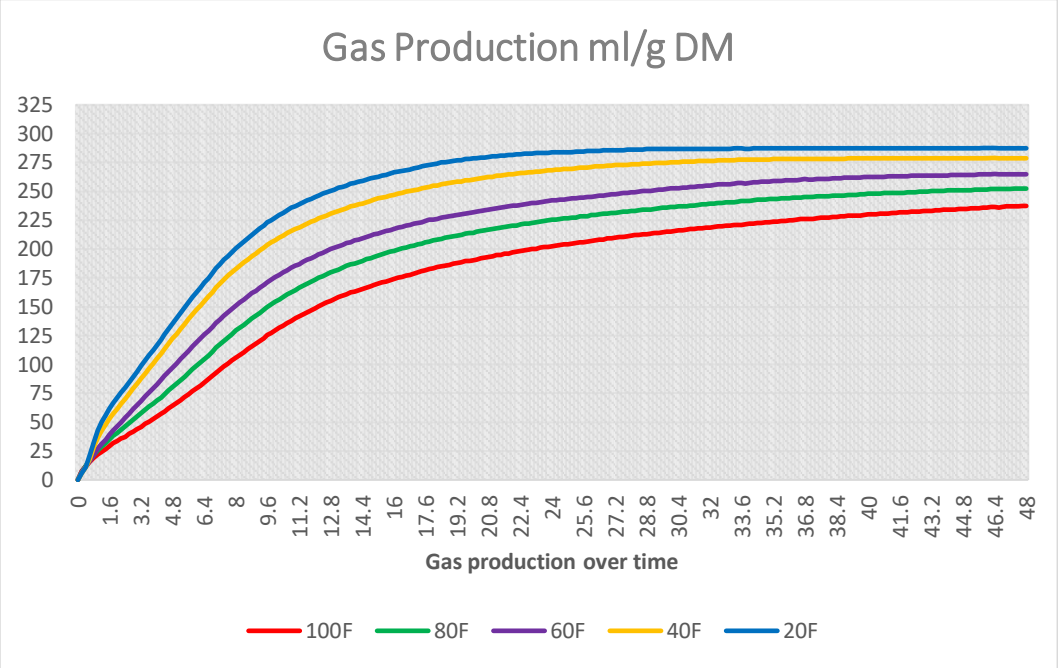

Figure 5. Gas production recorded automatically over a 48-hour interval across the five treatments with different Forage-to-Concentrate ratios.

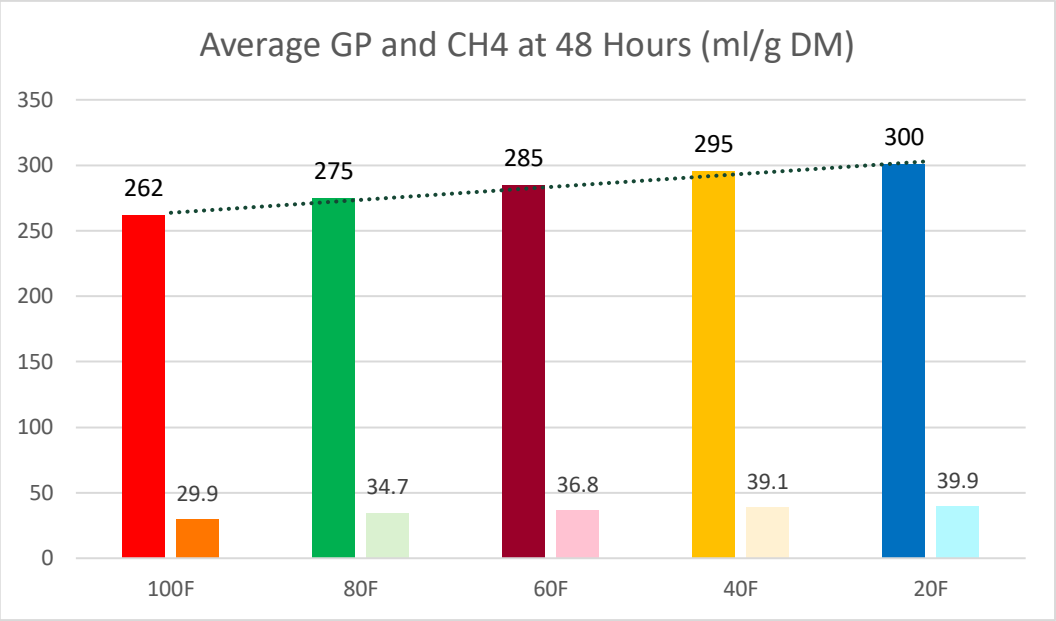

Figure 6. Comparison of the average of gas and CH4 production over a 48-hour interval.

The concentration of VFA after 48-hour incubation exhibited evident shifts across treatments, reinforcing the observed fermentation pattern changes (Figure 7). The total VFA concentration did not differ significantly among treatments ($P = 0.492$). However, acetate and butyrate concentration varied with F:C ratios (respectively, $P < 0.001$ and $P = 0.002$), whereas propionate proportion was not affected by any treatment

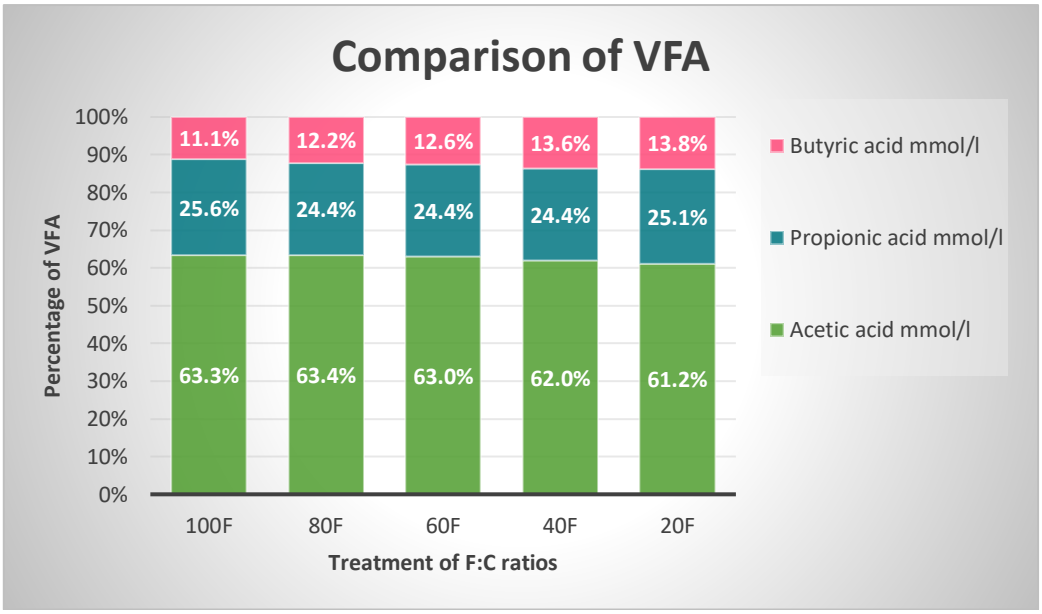

Figure 7. Comparison of VFA after 48-hour interval.

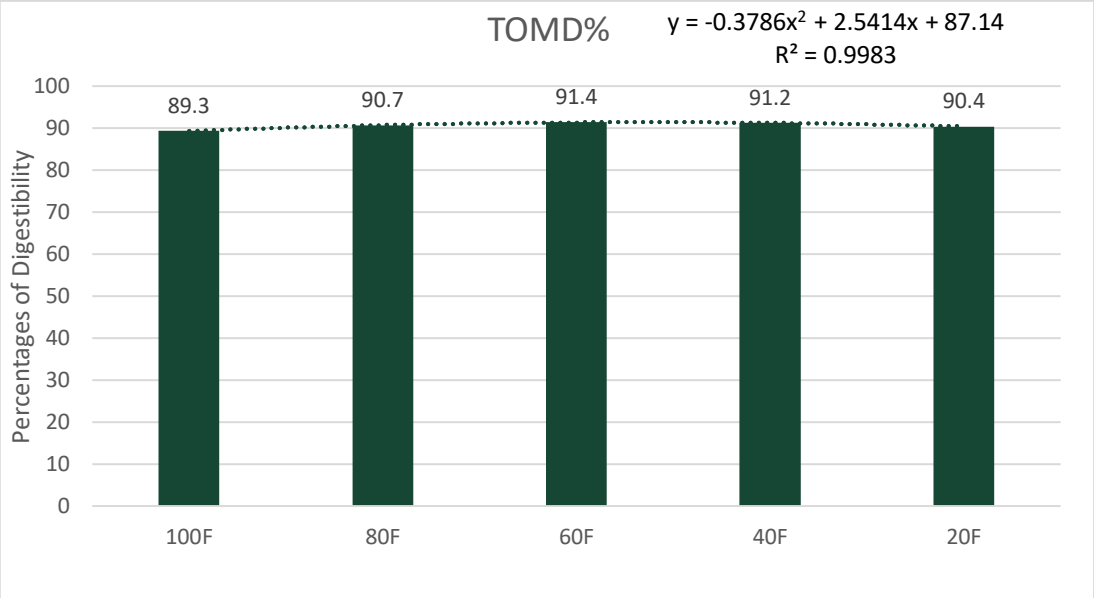

Figure 8. Total organic matter digestibility over the 48 hours incubation period of TOMD%.

The total dry matter digestibility (TDMD%) remained consistently high, fluctuating slightly. The statistical analysis revealed a p-value of 0.009, with a significant linear (P = 0.023) and quadratic (P = 0.003) relation.

Total Organic digestibility followed a similar trend (P = 0.01). The p a linear response (P = 0.006), and quadratic effect (P = 0.001) (Table 3).

4. Discussion

The study focused on how different F:C ratios affect the production of gases, particularly CH_4, as well as digestibility and VFA profiles in an in vitro system. The study was testing several F:C ratios: 100% F, 80% F, 60% F, 40% F, and 20% F. The study found that the production of both total gas and CH_4 varied across these different feeds. As the concentrate in the feed increased, there was a slight decrease on the pH, indicating a change in fermentation, which was supported by the statistical analysis of total gas output.

The finding demonstrated that the F:C ratios significantly influence gas production, CH_4 emissions, fermentation characteristics, and digestibility parameters in dairy cow feed which it will be discussed further in each part.

Higher concentrate levels led to increased total gas and CH_4 production, with a linear and quadratic response, emphasizing the role of diet composition in rumen fermentation dynamics. The observed changes in VFA profiles indicated a shift towards a more glucogenic fermentation pathway, as acetic acid concentrations declined while butyric acid increased with higher concentrate inclusion.

Despite the overall stability in total VFA concentration, the reduction in acetate-to-propionate ratio highlights the metabolic shift that could enhance energy availability for milk production but also pose risks such as subacute ruminal acidosis (SARA). The ruminal pH showed a slight decline with increasing concentrate levels, reflecting the impact of rapid starch fermentation and potential challenges in maintaining rumen buffering capacity.

However, in vitro results do not always translate directly to in vivo outcome due to inherent differences between laboratory setting and complex rumen environment, and the fact that in vitro is a closed system with no passage and flow as in the rumen. In addition to this, Fant and Ramin (2024) indicated that in vitro CH4 prediction systems are useful for screening feed ingredients, but it may overestimate or underestimate certain feed.

Regarding digestibility parameters, including TDMD% and TOMD%, exhibited a significant response to diet composition, with moderate concentrate inclusion (60% F and 40% F) optimizing nutrient utilization. However, excessive concentrate levels could negatively impact fiber digestion due to reduced ruminal pH.

4.1 Gas and methane production

Over 48 hours of incubation, there were clear changes in total gas and CH_4 production depending on the F:C ratios incubated. When the diet had a higher proportion of forage, it led to less gas production, whereas a higher concentrate content resulted in the highest gas being produced, as described by Kim et al.

(2018). A study by Benchaar et al. (2001) and Azmi et al. (2020) found similar results, showing that diets with higher concentrate levels produce a larger volume of total gas. This greater gas production in diets with higher concentrate levels is likely due to the intensified fermentation of non-structural carbohydrates, which produce more H_2 and CO_2 as byproducts of fermentation (Ramos et al. 2021), which are subsequently utilized by archaea bacteria by producing CH_4.

The comparative analysis of CH_4 mitigation strategies highlights the complementary roles of both in vivo and in vitro method in ruminant nutrition research. In vitro gas production methods provides a cost-effective, ethical, and scalable method for screening multiple dietary feeds, enabling precise control over experimental variables (Ramin & Huhtanen 2012). However, in vitro systems often oversimplify the complex rumen environment, leading to over- or underestimation of CH_4 emission, particularly for diets with high concentrate content (Meale et al. 2012; Raffrenato et al. 2018). These limitations emphasize the necessity of validating in vitro findings through in vivo trials (Danielsson et al. 2017; Klop et al. 2017).

The combination of in vitro and in vivo methods could bridge methodological gaps. Study by Fant and Ramin (2024) highlighted the predictive value of in vitro methods for preliminary assessments of dietary strategies, with subsequent validation needed through in vivo approaches. Nevertheless, discrepancies in CH_4 measurements between in vitro predictions and in vivo observations underline the need for standardized methodologies to enhance the reliability of research findings. Advances in measurement systems, such as combining in vitro methane prediction with GreenFeed or respiration chamber results, could optimize CH_4 mitigation research (Tedeschi et al. 2022; Ma et al. 2024).

Future research should prioritize the refinement of integrative methodologies to leverage the strengths of both systems, ensuring more reliable and scalable solutions for CH_4 mitigation. These efforts would contribute to sustainable ruminant production by balancing feed efficiency and environmental concerns.
In fact, apart from gas production, increasing concentrate proportion also resulted in an increase in CH_4 production. Methane production followed a similar trend to gas production, an increase of CH_4 production when the proportion of concentrate in the diet increases, as reported by Serment et al. 2016.

Methane production is usually associated with the digestion of fiber, whereas the rise in CH_4 production with more concentrate in the diet is likely due to enhanced microbial fermentation of rapidly digestible feed (Johnson & Johnson 1995). In addition, the increase in CH_4 production may depend on the fact that the in vitro system used is a closed system, and there is no passage rate or absorption of nutrients via the rumen wall.

In simpler terms, the in vitro setup, which is a controlled lab environment, can cause CH4 levels to appear higher than they would be in a cow's rumen. Normally, in the rumen, gases like CH_4 are affected by the rate of passage, absorption of nutrients in

the rumen wall, or used by microbes. However, in the lab, the setup is more like a sealed jar where gases collect and cannot escape. Therefore, the CH_4 levels measured in this environment might be higher than they would be in the rumen. This difference has been pointed out by Getachew et al. (1998), who noted that closed in vitro systems do not mimic the dynamic gas turnover occurring in the rumen, potentially leading to higher gas and CH_4 measurements.

4.2 Fermentation Characteristic

The fermentation profile revealed significant variation in individual volatile fatty acids (VFA) concentration, although the total VFA concentration remained stable across all the treatments, as described by Serment et al. (2010). Similar results were reported by J.C. Plaizer et al. (2018) and Olijhoek et al. (2022), in which the acetic acid concentrations decreased linearly with increasing concentrate levels, whereas butyric acid concentration increased. Propionic acid concentration did not show a significant overall change across the different dietary treatments, but the observed quadratic trend suggests a nonlinear response to diet composition.

This pattern may indicate that moderate adjustments in the diet can slightly reduce propionate production, while more extreme changes may reverse this effect. Propionic acid is a key gluconeogenic precursor essential for ruminant energy metabolism (Bergman, 1990; Aschenbach et al., 2011), so its relative stability across treatments implies that fermentable substrates remained sufficient to sustain consistent propionate synthesis. This contrasts with the significant linear increases observed in total gas and methane production, reflecting how different fermentation pathways respond uniquely to dietary variation (Janssen 2010). Understanding these nonlinear effects on propionate production can aid in optimizing feed formulations to maintain animal energy supply while mitigating methane emissions.

A previous study by Agle et al. (2010) also indicates that higher forage diets promote acetate production, while high-concentrate diets enhance propionate and butyrate production due to increase of starch. This shift occurs because the fermentation of structural carbohydrates in forage primarily produce acetate, while the rapid fermentation of starch from concentrates generates more propionates and butyrate (Trotta et al. 2018). In our study, an increased in concentrate proportion did not affect propionate concentration, that remained unchanged between treatments, in contrast with what reported by other authors (Kim et al. 2018; Vera et al. 2025). The ruminal pH exhibited a slight decline with an increasing concentrate ratio, remaining in physiological ranges .

This decline is likely due to the rapid fermentation of starch, leading to increase of VFA production and reduce buffering capacity in the rumen (Zebeli et al. 2008). The pH reduction observed in this study aligns with previous finding that have reported a similar trend in response to higher concentrate inclusion (Zebeli et al. 2008; Trotta et al. 2018). However, it must be mentioned that in vitro studies show a greater pH stability attributable to the inclusion of buffer systems within the inoculum (Serment et al., 2016).

4.3 Digestibility

The higher concentrate levels in ruminant diets are generally associated with increased digestibility due to lower fiber content and higher fermentability (Chen et al. 2021). The result of this in vitro study observed the highest TDM% and TOMD% at 60 F, probably due to the balanced diet, supplying readily usable substances for the microbial community, boosted their function and also aided in the breakdown of fibre (Getachew et al. 2005).

In addition, the higher digestibility observed with 60 F may be because the donor cows were already fed a 60:40 F:C diet. Consequently, the rumen microbiome had already adapted to this diet composition.

However, excessive concentrate inclusion can negatively impact the rumen function. High level of carbohydrates can drive the rumen pH to drop, creating an unfavorable environment for fiber digesting bacteria negatively affecting fiber digestibility (Olijhoek et al. 2022). This could explain the slight increase on OM digestibility for 40F and 20F. These findings highlight the importance of balancing F:C ratios to maximize digestibility while minimizing negative impact on the rumen function.

During the protein digestion, NH_3-N is produced, serving as precursor for microbial protein synthesis. There was a linear relationship between the amount of concentrated feed in the diets and the resulting level of ammonia nitrogen (NH3-N). This suggests that there was an increase of protein degradation even though all the diets were balanced to provide similar crude protein level. Other studies reported negative effects Egle et al. (2010) or not significant effects Vera et al. (2025) regarding the addition of concentrate in the diets and NH_3-N production.

4.4 Limitation

This study offers valuable insights into the effects of F:C ratios on fermentation dynamics, total gas and CH_4 production, and digestibility. However, several limitations should be acknowledged. The experiment was conducted under controlled in vitro conditions, which may not fully replicate in vivo fermentation dynamics. Additionally, microbial composition and adaptation were not assessed, potentially influencing gas production results. Furthermore, in vitro systems do not account for absorption rates, which may affect total gas production over the incubation period. This limitation is especially important when evaluating high-concentrate diets, as in vitro systems do not accurately replicate the dynamic absorption processes of the rumen. Unlike in vivo conditions, where fermentation byproducts such as volatile fatty acids are continuously absorbed, in vitro setups allow these compounds to accumulate unnaturally. This buildup can inhibit microbial activity and lead to an overestimation of gas production, making the

results less representative of actual rumen function in high concentrate feeding scenarios.

5. Conclusion

In conclusion, while increasing concentrate levels- up to a certain point- enhanced digestibility due to improved fermentability, the results of this study emphasize the need for a balanced approach to concentrate inclusion, ensuring optimal digestibility without compromising rumen function.

While the findings provide valuable insights into the effects of F:C ratios on rumen fermentation, the study's limitations should be considered. The in vitro nature of the experiment may not fully replicate in vivo fermentation dynamics, and microbial composition was not assessed. Future studies should focus on long-term in vivo trials to better understand the implications of dietary interventions on animal performance, feed intake, and methane mitigation strategies.

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Popular science summary

Cows produce methane (a greenhouse gas) during digestion, mainly when breaking down feed in the rumen—a part of their stomach.

This study looked at how changing the balance between forage (like grass) and concentrate (like starch-rich feed) in dairy cow diets affects how much total gas and methane they produce, and how well they digest their food. We tested five different feed combinations, from 100% forage to 20% forage, using an artificial rumen system in the lab. We measured how much gas and methane was produced, how acidic the rumen environment became, and how well the feed was broken down.

We found that adding more concentrate to the diet increased gas and methane production and slightly lowered rumen pH. Interestingly, the best digestion occurred with a balanced mix of about 60% forage, suggesting cows use their feed more efficiently at this ratio. While high-concentrate diets are often thought to reduce methane, our results—and those of other studies—show this isn't always the case. In some situations, more fermentation can still lead to higher methane. These findings show that adjusting cow diets can help reduce emissions and improve feed efficiency, benefiting both farmers and the environment.

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