Effect of spruce (*Picea abies)* biochar on ruminal dry matter digestibility and methane production *in vitro*

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Author:
Tatiana Konkova

Supervisor:
Anni Halmemies- Beauchet-Filleau

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Abstract

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Faculty of Agriculture and Forestry	Department of Agricultural Sciences

Tekijä — Författare — Author

Tatiana Konkova

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Tiivistelmä — Referat — Abstract

Biochar, produced by heating biomass in oxygen-limited environments, is known for its potential to reduce methane emissions, by altering rumen fermentation. However, the literature lacks exploration on how biochar affects nutrient digestibility and ruminal methane production across various diets. Therefore, we examined the effects of spruce (*Picea abies*) biochar on several forage plant species options and forage-to-concentrate (F:C) ratios.

Two in vitro trials of 24 hours were conducted with Gas Endeavour® equipment at Animal Science laboratory of the University of Helsinki. There were three rumen fluid donor animals and the ratio of rumen fluid and McDougall's buffer was 1:2 (vol:vol). The first trial tested biochar levels (0%, 0.25%, and 0.5% of diet dry matter (DM)) with four silage options that were pure grass (*Phleum pratense – Festuca pratensis*), grass mixed 1:1 in DM with red clover (*Trifolium pratense*), faba bean (*Vicia faba*), or maize (*Zea mays L*) at a fixed F:C ratio of 65:35. The concentrate consisted of oats and rapeseed meal. The second trial explored the effects of the biochar (0.5% of diet DM) and an alternative methane inhibitor (product X) alone or combined across 65:35 and 45:55 F:C ratios. Key parameters such as total gas and methane production, DM digestibility, rumen fluid pH, and volatile fatty acid (VFA) concentrations were analyzed.

Grass silage (D-value 669, neutral detergent fiber (NDF) 523 g/kg DM) and red clover silage (D-value 626, NDF 362 g/kg DM) were harvested from the first cut. Maize silage was more digestible than faba bean silage (D-value 679 vs. 593 g/kg DM) and contained more starch (278 vs. 48 g/kg DM). High forage diet (65:35) had higher NDF content (453 vs 407 g/kg DM) and lower starch content (114 vs 179 g/kg DM) than low forage diet (45:55). Biochar's effect on DM digestibility and the production of rumen methane or carbon dioxide was not significant across different silage plant species and F:C ratios. Grass silage led to lower total methane production compared to silage mixtures ($p<0.05$), and the combination of biochar and product X in a low forage diet numerically reduced gas production. However, when methane production was calculated per digested DM, no differences were observed. Feeding maize silage increased the total production of methane and carbon dioxide in the rumen compared to faba bean silage ($p<0.001$), but gas productions per digested DM remained unaffected. Biochar did not significantly affect final rumen pH across silage species. Biochar with grass silage linearly reduced the total VFA content of the rumen fluid ($p=0.003$) and had tendency for smaller molar proportion of acetic acid in VFA ($p= 0.075$). In the second trial biochar or product X didn´t have significant effect on rumen fermentation pattern across forage levels.

The effects of biochar, silage plant species and F:C ratios on *in vitro* rumen fermentation and methane production were minimal, despite differences in diet composition.

Avainsanat — Nyckelord — Keywords

Spruce biochar, forage species, F:C ratio, rumen fermentation, methane production, *in vitro*

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Dedication

This one's for my husband, whose endless support, patience, and encouragement have been my driving force. Couldn't have done it without you!

Abbreviations

DM = dry matter

VFA = volatile fatty acids

NDF = neutral detergent fiber

CP = crude protein

CFat = crude fat

CF = crude fibre

EE= ether extract (crude fat)

NFE = nitrogen free extract

ME = metabolizable energy

BC= biochar

PX = product x

GS= grass silage

RCS = red clover silage

FBS = faba bean silage

MS = maize silage

RSM = rapeseed meal

STP = standard temperature and pressure

TMR = total mixed ration

F:C = forage-to-concentrate ratio

1 Introduction

Ruminant livestock, with their unique digestive system, generate methane during microbial fermentation in the rumen. According to the European Environment Agency (2023), methane emissions from enteric fermentation is responsible for 48% of total agricultural greenhouse gas emissions in Europe. To mitigate these emissions, various strategies involving feeds and feed additives have been explored, aiming to reduce the environmental impact of livestock production without compromising animal health or productivity. Among these strategies, biochar has emerged as a noteworthy candidate due to its multifaceted benefits and potential to reduce methane emissions. Biochar is a carbon-rich material produced through the pyrolysis of biomass in an oxygen-limited environment. This process transforms organic materials into a stable form of carbon (Lehmann and Joseph, 2015). The substantial porosity and extensive surface area of biochar's structure may play important role affecting rumen fermentation, methane production and nutrient digestibility, through mechanisms such as gas absorption and alterations to microbial populations or their habitats (Hansen et al., 2012; Lehmann and Joseph, 2015). Additionally, biochar has been observed to reduce methane production by modifying nitrogen metabolism and the proportions of volatile fatty acids (VFAs) in the rumen (Leng et al., 2012ab; Saleem et al., 2018).

Current *in vitro* and *in vivo* studies lack exploration on how biochar affects nutrient digestibility and methane production in terms of various diets. Diet composition may significantly impact the rumen's microbial communities and fermentation patterns (Henderson et al., 2015; Neubauer et al., 2018), which may contribute to the biochar's effectiveness. Different forage-to-concentrate ratios and forage plant species can modify the predominant fermentation pathways (acetate, propionate, and butyrate production), directly affecting methane emissions (Bayat et al., 2017; Chowdhury et al., 2023). Biochar's interaction with these varying fermentation profiles (Saleem et al., 2018; Cabeza et al. 2018) could enhance or diminish its efficacy in methane mitigation. Therefore, exploring biochar's effects on various diets is essential for leveraging its potential as a methane mitigation feed additive, offering a practical solution for reducing livestock emissions. The aim of this study was to evaluate the effect of biochar on rumen dry matter digestibility and methane production *in vitro*, considering diets with both high and low forage-to-concentrate ratios and four silage species options cultivable in Finland.

2 Literature review

2.1 Biochar´s effect on methane production and DM digestibility (*in vitro* and *in vivo*)

The scientific literature presents varied findings on the effects of biochar on nutrient digestibility and rumen fermentation or methane production, which may be attributed to several factors including biochar pH, incubation duration, feed substrate, and biochar source (Table 1; Table 2).

Table 1 Effect of biochar on rumen fermentation and methane production *in vitro.*

Study references	Biochar			Incubated basal diet	Incubation time, h	VFAs		Methane production
	Source	Pyrolysis, °C and pH	Inclusion level, % in DM			Acetic	Propionic	
Benchaar et al. (2023)	rice husk; pine; forestry wood; forest biomass; spruce; black shavings; black spruce shavings	450 -500 7.3–7.7	1, 2, 5	TMR 50:50	24	no	no	no
Dung et al. (2022)	rice husk	700 8.9	1, 3, 5, 7	rice straw and concentrate 70:30	48	no	no	↓ 10-17%
Tamayo et al. (2021)	jack pine (3 types)	400-600 n/a	20	TMR (barley silage, barley grain and minerals) 60:40	48	no	no	no
Cabeza et al. (2018)	miscanthus straw; oil seed rape straw; rice husk; soft wood pellets; wheat straw	550-700 9.7–9.8	1, 10	hay, barley, rapeseed meal 50:50	24	no	↓	↓ 1-6%
Saleem et al. (2018)	pine	400-600 4.8	0.5, 1, 2	barley silage, barley grain, canola meal, supplements 60:40	48	↑	↑	↓ 25%
Leng et al. (2012)	rice husk	900-1000 n/a	0.5, 1, 2, 3.5	cassava root meal and urea	24, 48	n/a	n/a	↓ 10-13%
Hansen et al. (2012)	gasified; straw- based; wood-based; activated carbon	n/a n/a	9	hay and a mixed ration (MR) (50:50)	48	n/a	n/a	↓ 20%
Pereira et al. (2014)	corn stover pine wood chips	350-550 8.2–9.9	0.5, 1, 2, 5	Grass silage or hay	24	no	no	no

n/a, information is not available; no = no effect; ↑ = increase; ↓ = decrease.

In vitro studies, such as those by Saleem et al. (2018), Dung et al. (2022), and Leng et al. (2012a), have shown significant reductions in methane production with biochar supplementation (Table 1). However, studies conducted by Benchaar et al. (2023), Tamayo et al. (2021) and Cabeza et al. (2018) showed no effect of biochar either on rumen fermentation or methane production (Table 1).

Similar variability in the effects of biochar on methane production was also observed in *in vivo* studies (Table 2). Leng et al. (2012b) observed a significant reduction in methane emissions (22%) with biochar supplementation. Winders et al. (2019) noted a minor effect—a trend towards decreased methane production, especially with 0.8% biochar dose in diet DM, indicating a possible dose-dependent effect. In contrast, Terler et al. (2023) found no significant impact of biochar on methane emissions in dairy cows, whether supplemented with urea or not (Table 2). This difference *in vivo* studies is likely due to the complex dynamics of the live rumen ecosystem, including animal physiological factors and variations in diet and management practices (Qomariyah et al. 2023).

The impact of biochar on nutrient digestibility is also variable. Saleem et al. (2018) found that biochar did not adversely affect organic matter digestibility *in vitro*, suggesting its neutral impact on nutrient availability. However, Dung et al. (2022) reported that while low levels of biochar (1% or 3% DM) did not affect digestibility, higher levels (5% or 7% DM) reduced OM and DM digestibility, indicating a dose-dependent effect on nutrient availability. *In vivo* studies, such as those by Erickson et al. (2011) and Winders et al. 2019, have shown that biochar supplementation does not consistently affect dry matter intake or overall diet digestibility. These findings suggest that while biochar can reduce methane emissions, its impact on the digestibility of major nutrients (DM, OM) may not be significant, thereby not compromising animal performance.

Overall, the differential outcomes of *in vitro* and *in vivo* studies on biochar's effect underscore the complexity of its application as a mitigation strategy. *In vitro* studies, while providing controlled environments to understand biochar's potential mechanisms, cannot fully replicate the intricate interactions within a live animal's rumen and overall physiology. Conversely, *in vivo* studies reflect the real-world complexity of biochar supplementation, considering the entirety of animal responses and environmental interactions.

Table 2 Effect of biochar on rumen fermentation and methane production *in vivo*.

Study references	Biochar			Basal diet	Species	Trial time	Methane production
	Source	Inclusion level, % in DM	Pyrolysis °C, pH				
Cattle							
Leng et al. (2012b)	rice husks	0.6	> 400 n/a	cassava root chips (ad libitum) supplemented with fresh cassava foliage at 1% of LW	12 Lao "Yellow" beef cattle LW 80 - 100 kg	98 days including 21 days for adaptation	↓ 22 %
Sengsouly and Preston (2016)	rice husks	1	950	Ensiled cassava root (ad libitum) supplemented with urea (2% of ensiled root DM); fresh cassava foliage at 25% of the DM of the basal diet, and rice straw at 1% of live weight	12 Lao "Yellow" beef cattle LW 80 - 100 kg	120 days including 15 days for adaptation	No effect
Winders et al. (2019)	pine wood	0, 0.8, 3	n/a 8.0	70:30 (brome hay and wheat straw/or dry rolled corn corn silage, wet distillers grains plus solubles, supplement)	Six crossbred steers LW 529 kg	93 days (6 periods (14-24d), each period includes min. 8 days for adaptation)	↓ 9-18% for biochar level 0.8%
Terler et al. (2023)	pure ash wood	20	500 10.1	Fresh forage ad libitum (mixture grass- maize silage and hay) and 5 kg concentrates (barley, maize, wheat, sugar beet pulp, soybean meal, wheat bran, rapeseed oil)	18 dairy cows (Holstein Friesian and Simmental) Lactation – av. 110 days LW – av. 659 kg	105 days Include 2 weeks for adaptation	No effect
Goat							
Silivong and Preston (2016)	rice husks	1	n/a n/a	Bauhinia acuminata (ad libitum) and molasses; 70% Bauhinia acuminata and 30% water spinach	16 Lao local goat	97 days, including the 15 days adaptation period	No effect
Hang et al. (2019)	rice husks	0, 0.5, 1, 1.3	950 n/a	Ad libitum fresh cassava foliage (sweet variety) supplemented with 4% (DM basis) of ensiled brewers' grain	12 Bach Thao goats	94 days including 15 days for adaptation	↓ 15-39%

n/a, information is not available; ↓ = decrease.

This variability in study outcomes highlights the need for a holistic approach to research, combining both *in vitro* and *in vivo* methodologies to tailor biochar

supplementation. Despite the notable progress in biochar research, significant gap remains in the literature. Specifically, the effects of feed substrates, including different forage plant species and forage-to-concentrate ratios, on biochar's impact on methanogenesis and nutrient digestibility remain underexplored, indicating a need for more focused research in this area.

2.2 Forage plant species and its effect on methane production

Different plant species used for silage exhibit varying compositions of fiber and starch, that may influence rumen fermentation and subsequent methane production. Grass silages (*Phleum pratense – Festuca pratensis*) typically displays moderate to high fiber levels, while legume silages like red clover (*Trifolium pratense*) and faba bean (*Vicia faba*) tend to have lower fiber and higher protein content (Kuoppala et al., 2009; Chowdhury et al., 2023). Diets high in grass silage tend to result in higher ruminal pH and a lower proportion of propionate among VFAs. High fiber diets promote acetate and butyrate production in the rumen (Wang et al. 2020). Since acetate and butyrate productions are closely linked with methane formation (Moss et al. 2000), high-fiber diets generally lead to higher methane emissions. The slower fermentation rate of fiber also means a longer retention time in the rumen, providing more opportunity for methane-producing microbes to act (Terry et al., 2019; Wang et al., 2020). Maize silage (*Zea mays L*) usually contains higher starch levels compared to other silages, with grass and forage legume silages having minimal starch content. Diets with the high starch content tend to promote a VFA profile dominated by propionate (Chowdhury et al., 2023). Propionate production competes with methane formation pathways in the rumen, thus potentially leading to lower methane emissions (Ungerfeld., 2020). The rapid fermentation of starch also reduces the time feed spends in the rumen, limiting methane production opportunities (Squara et al., 2022).

2.3 Forage-to-concentrate ratio and its effect on methane production

The forage-to-concentrate ratio (F:C) in ruminants' diets significantly influences methane production. High forage proportions, typically associated with increased fiber, lead to higher methane emissions due to enhanced acetate and butyrate pathways in rumen fermentation. For instance, increasing the F:C ratio from 47:53 to 68:32 in dairy cow diets has been shown to increase methane emissions linearly, reflecting the higher fiber content's impact on rumen fermentation patterns (Aguerre et al., 2011). Conversely, diets with higher concentrate proportions, which are richer in

starch, tend to decrease methane emissions. This is attributed to the promotion of propionate production in the rumen, which serves as an alternative H_2 sink to methane formation. For example, a study on dairy cows (Nordic Red) showed that high-concentrate diets (65%) resulted in increased propionate and butyrate ratios within the total VFAs, while reducing the acetate-to-propionate ratio compared to low-concentrate diets (35%). This shift in rumen fermentation led to decreased methane emissions; high-concentrate diets showed a 13.5% reduction in methane production compared to low-concentrate diets (Bayat et al., 2017).

3 Study objectives and hypotheses

The preliminary trial aimed to examine the potential of increasing spruce biochar levels (0%, 0.1%, 0.25%, 0.5%, 1% and 2% in DM) to reduce ruminal gas production *in vitro* without compromising digestibility. We hypothesized that the reduction in methane production is dependent on biochar inclusion level and at very high inclusion levels DM digestibility may be impaired.

The objective of Trial 1 was to investigate the varying effects of different concentrations of spruce biochar (0%, 0.25%, and 0.5% in DM) on total gas and methane production, as well as diet digestibility, within different basal forage options.

Hypotheses:

- Biochar is anticipated to alter rumen fermentation by promoting the production of propionate, potentially leading to decreased methane production without negatively affecting dry matter digestibility.
- The reduction in methane emission due to biochar is dose-dependent.
- The effect of biochar will vary depending on basal diet.
- Forages rich in fibre promote and those rich in starch mitigate ruminal methane formation.

The objective of Trial 2 was to compare the effects of two potential methane inhibitors biochar and an alternative product (product X) individually and in combination, considering two different concentrate levels (55% and 35% of diet DM) on rumen gas production and diet dry matter digestibility *in vitro*.

Hypotheses:

- Biochar and product X are expected to decreased methane production, with more pronounced effect for the combination of biochar and product X than when both these products are delivered separately, due to their different methane mitigating mechanisms. In addition, the products are not expected to effect on dry matter digestibility.
- A high forage diet is expected to result in a higher total methane production due to its higher fiber and lower starch content.

This thesis is focused on the effects of forage plant species and F:C ratios in combination with spruce biochar on ruminal fermentation and methane production, whereas individual effects of forage plant species and F:C ratios on methane production are less thoroughly discussed.

4 Materials and Methods

4.1 Experimental set-up and design

The research was conducted in Finland at the University of Helsinki Viikki research farm and adjacent Animal Science laboratory in the period April-May 2023. The project consisted of three *in vitro* trials. The preliminary trial screened the most efficient dosages of biochar to mitigate ruminal methane production *in vitro* for the subsequent experiments. Then, the first trial focused on investigating the effects of two different dosages of biochar under various basal diet options on ruminal methane production *in vitro*. Finally, the second trial compared the effects of product X, and biochar alone or combined on *in vitro* methane production at two different concentrate levels. The automatic gas flow measuring system Gas Endeavour® (Bioprocess Control, Lund, Sweden) was employed to measure methane and total gas production while monitoring their flow throughout the 24-hour incubation period (Figures 1 and 3).

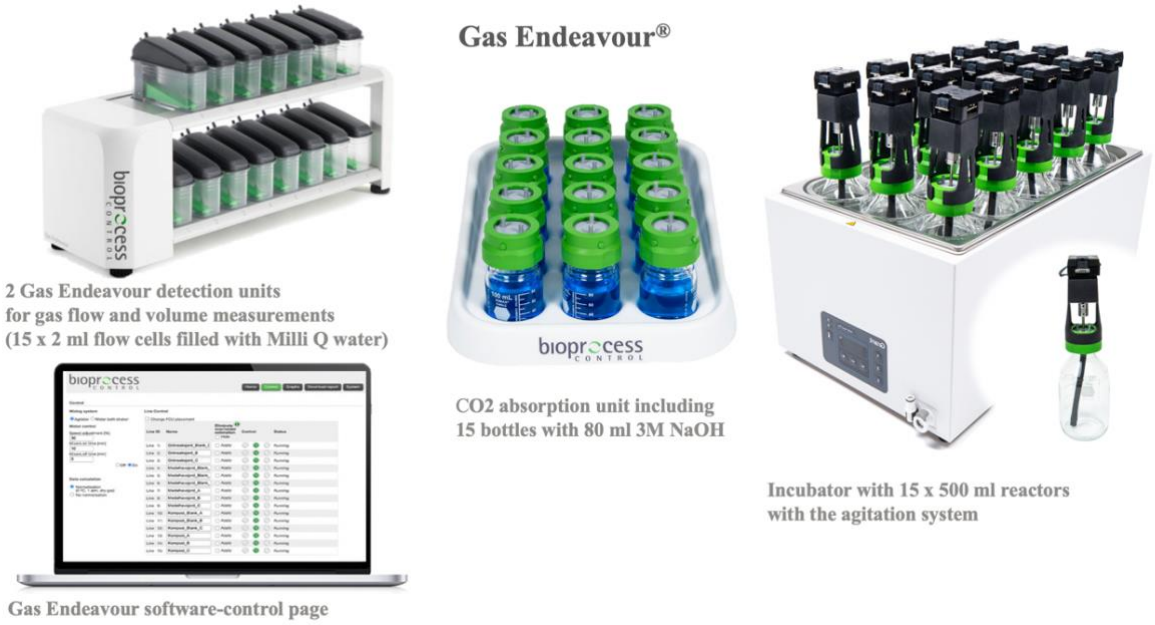

Figure 1 Units of the automatic gas flow measuring system Gas Endeavour® retrieved from https://www.bioprocesscontrol.com/products/gas-endeavour/

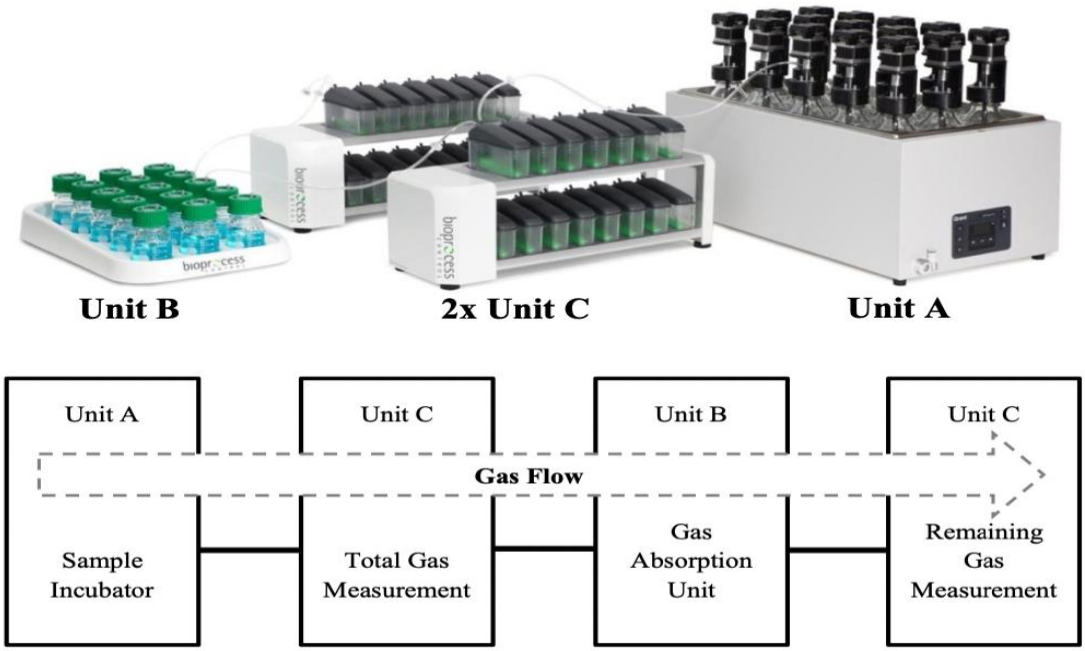

Figure 2 Gas Endeavour® system units. Bioprocess Control, Lund, Sweden. (2022). Gas Endeavour® Retrieved from https://bpcinstruments.com/wp-content/uploads/2022/02/2022_Gas-Endeavour-Manual.pdf

The Gas Endeavour® system comprises three main units, namely A, B, and C, each dedicated to specific functions (Figure 2):

- **Unit A** (Sample Incubation Unit): Within this unit, up to 15 test vessels containing feed samples with suitable microbial inoculum were incubated at a desired temperature using a thermostatic water bath and 15 x 500 ml glass bottles. The media in each vessel underwent continuous mixing through a slow rotating agitator, facilitating a sustained gas production from the material.
- **Unit B** (Gas-Absorption Unit): The gas produced in each vial from Unit A passed through individual vials containing solutions capable of absorbing specific gas fractions. The gas was led through an alkaline solution, such as NaOH, which selectively retained acid fractions like CO_2 and H_2S, allowing only methane (and remaining traces like H_2) to pass through to the gas monitoring unit.
- **Unit C** (Gas Volume Measuring Device): In this unit, the volume of gas released from Unit A or Unit B was individually measured using a wet gas flow measuring device with a multi-flow cell arrangement (15 cells). The device operated based on the principles of liquid displacement and buoyancy, precisely monitoring

ultra-low gas flows. Upon the flow of a defined volume of gas (2 ml) through the flow cells, a digital pulse was generated. The system integrated an embedded data acquisition system, which recorded, displayed, and analyzed the results.

Figure 3 Adjusted Gas Endeavour® at Helsinki University Laboratory (Photo: Tatiana Konkova, HU)

Opposite to preliminary trial, experiments 1 and 2 followed the same design, featuring 5-6 laboratory replicates (5-6 gas runs of 24h incubation) per experiment (Table 1). To serve as control, pure grass silage was included in all runs at the midmost vessel 8. Furthermore, two blinds were in the first and last vessels, 1 and 15 respectively. The blinds containing only rumen fluid and artificial saliva (McDougall,1948) in proportion 1:2 (in vol: vol) were included in all runs and used to take into account the background fermentation and nutrients from inoculum. Treatments (3.78 g of DM) were randomly allocated to vessels 2-7 and 9-14 at every run.

4.2 Experimental feeds and treatments

The treatments in experimental trials are presented in Table 3. The basal diet for the preliminary trial contained 65% in DM of grass silage and 35 % of concentrates (30% of oats and 5% of rapeseed meal). On the top of the basal diet was added 6 different dosages of biochar, 0, 0,1%, 0,25%, 0,5%, 1% and 2%. There were 2 replicates for each of biochar dosages within the one gas run.

Table 3 Experimental trials and treatments

Experiment	Treatment (% of diet DM)		Inhibitors (% of diet DM) on the top of diet	Amount of 24h gas runs per trial
	Forages %	Concentrates %		
Preliminary	GS 65	Oats 30 + RSM 5	BC 0; 0.1; 0.25; 0.5; 1.0; 2.0	1
Trial 1	GS 65 GS 32.5 + MS 32.5 GS 32.5 + RCS 32.5 GS 32.5 + FBS 32.5	Oats 30 + RSM 5	BC 0; 0.25; 0.5	5
Trial 2	GS 65 or GS 45	Oats 30 + RSM 5 or Oats 47.2 + RSM 7.8	BC 0.5; PX 1.35; PX 1.35 + BC 0.5	6

DM, dry matter; GS, grass silage; MS, maize silage; RCS, red clover silage; FBS, faba bean silage; RSM, rapeseed meal; BC, biochar; PX, product X

In the first trial, the basal diet consisted of 65% of forage, and 30% of oats and 5% of rapeseed meal as concentrates of diet DM. The 65% of forage comprised only grass silage, or mixtures of grass silage with maize, red clover or faba bean silage (1:1 in DM basis). Each of these basal forage options was supplemented with increasing levels of spruce biochar, specifically 0%, 0.25%, or 0.5% in DM.

In the second trial, the basal diet DM consisted of either 45% or 65% of grass silage alone, with the remaining proportions being 55% or 35% of oats and rapeseed meal as concentrates respectively. Each of these two basal diet options was investigated alone (grass silage 45% and grass silage 65%) or supplemented with 0.5% of diet DM spruce biochar, or with 1.35% of product X, and with a combination of 1.35 % of product X and 0.5% BC.

The physical properties and chemical composition of biochar (Carbofex Ltd., Nokia, Finland) are provided in Table 4. Biochar was produced through the pyrolysis of spruce material at a temperature range of 650-700°C, and its particle size distribution ranged from 0 to 20 mm. The European Biochar Certificate (EBC) certificate of spruce biochar is attached as appendix 1.

Product X is a commercial product currently under development, and as such, we are unable to disclose any information regarding its composition or properties. By including product X in our study (trial 2) we designed to assess whether product X shows any effects distinct from those of biochar, which constitutes the primary focus of the study.

Table 4 Physical properties and chemical composition of spruce biochar (Carbofex Ltd.)

Parametrs	Unit, db	Value
DM	%	76.8
Specific surface area	m2/g	398.6
Watert holding capacity < 2 mm	%	318.0
Ash content (550°C)	%	1.7
Total carbon	%	95.2
Hydrogen	%	0.8
Nitrogen	%	0.7
Sulfur	%	< 0.03
Oxygen	%	2.1
pH		9.5

Table based on Eurofins test report 2023 (AR-23-FR-012882-01); db - dry basis

All silages swards were grown and harvested at Viikki research farm. Their chemical composition is provided in Table 5. The grass silage utilized in this study was prepared form a mixture of timothy-meadow fescue *(Phleum pratense – Festuca pratensis)* sward. The first-cut grass silage was cut with mower-conditioner (Krone EasyCut 3210 CV, Maschinenfabrik Bernard Krone GmbH, Spelle, Germany), pre-wilted, and harvested with self-loading forage wagon (Krone XXL R/GL, Maschinenfabrik Bernard Krone GmbH) in bunker silos using AIV Pro NC (Eastman Inc, Oulu, Finland) as the ensiling agent (5L/ton of feed).

Table 5 Chemical composition of incubated feeds used in experiments

	GS	RCS	MS	FBS	Oats	RSM	BC	PX
Dry matter, g/kg	945	924	941	936	943	947	802	993
Composition, g/kg DM								
Ash	95.4	115	39.5	115	34.9	75.1	na	na
Crude protein	179	188	78.9	180	147	375	na	na
Total fat	35.0	23.0	18.0	15.9	45.9	30.5	na	na
NDFom	533	362	399	359	313	254	na	na
Starch	nd	2.20	278	47.8	380	nd	na	na
ME, MJ/kg DM		10.0	10.9	9.52	11.3	11.2	na	na

DM, dry matter; na, not applicable; nd, not determined; NDFom, ash free NDF; ME, metabolized energy; GS, grass silage; MS, maize silage; RCS, red clover silage; FBS, faba bean silage; RSM,rapeseed meal; BC, biochar; PX, product X

As a difference to grass silage, red clover (*Trifolium pratense*, first cut) and faba bean (*Vicia faba*) were ensiled in big bales (Lely Welger RPC 245 Tornado, Lely International, Maassluis, the Netherlands) using AIV2 Plus Na (Eastman Inc, Oulu, Finland) at the dosage 5-L/ton of feed. Maize silage (*Zea mays L*) used in the 1st trial was cut at late stage with Claas Jaguar 950 forage harvester (Claas KGaA GmbH, Harsewinkel, Germany) and harvested with self-loading forage wagon (Krone XXL R/GL, Maschinenfabrik Bernard Krone GmbH) in a bunker silo. The preservation agent was Kofasil Ultra K (ADDCON GmbH, Bitterfeld-Wolfen, Germany), at a dosage of 4 l/ton of feed. At the time of harvesting, the DM content of the maize was approximately 31%, and the starch content was 26% on a DM basis. The concentrates feed used in this study consisted of oats (*Avena sativa*) grown and harvested at Viikki research farm and commercially produced rapeseed meal (*Brassica napus L.*) from Lantmännen Agro Ltd. The nutritional value of the diets presented in the table 6.

Table 6 Nutritional value of diets used in experiments 1 and 2

	Trial 1 and 2	Trial 2	Trial 1	Trial 1	Trial 1
In diet DM, g/kg DM	Grass 65	Grass 45	Red clover	Maize	Faba bean
Ash	76.2	65.3	82.5	58.1	82.5
CP	179	180	182	147	180
Total fat	38.0	39.7	34.2	32.5	31.8
NDF	453	407	397	410	396
Starch	114	179	115	204	130

DM, dry matter; NDF, neutral detergent fiber; CP, crude protein, Grass diet 65, forage 65 % in diet DM (experiment 1 and 2); Grass diet 45, forage 45 % in diet DM (experiments 2)

4.3 Rumen Inoculum

In order to prioritize animal welfare, the trial was conducted in accordance with Directive 2010/63/EU, and Finnish Act on the Protection of Animals Used for Scientific or Educational Purposes (497/2013), the primary laws governing animal experimentation. All necessary measures were taken to ensure that the animals involved in the trial were handled and cared for appropriately.

Three multiparous, rumen fistulated Nordic Red cows were used as rumen inoculum donors for all experiments. They were housed in tie-stalls at the barn of Viikki research farm and started to consume a standard diet 14 d prior to experiments. Cows were offered ad libitum access to silage and 150 g/d of minerals and vitamins (Seleeni–E–Melli TMR mineral, manufacturer Lantmännen Feed, Turku, Finland) scattered over fresh silage in the morning. The silage was distributed twice daily at 9.30 and 20.30 h. The silage contained 88.4% grass species, 10.9% red clover and 0.64% weeds in DM. It was harvested at 1st cut at fields of Viikki research farm and ensiled in big bales using AIV2 Plus Na at the dosage 5L/ton of feed. Average silage daily intake for whole period (pretrial, trial 1 and trial 2) was 23,4 kg/DM/day. Additionally, cows were fed 4 times daily with 2.5 kg of concentrates (8,8 kg/DM/day). The concentrate pellet was produced by Hankkija Oy (Kotka factory, Finland) and contained rapeseed meal, barley, wheat, molassed sugarbeet pulp, mixed molasses, some by-products of cereals, and vitamins and mineral. Cows had free access to water and salt blocks, and were milked daily at 6.30 and 17.00. Rumen inoculum was collected between 9.00 and 9.30 h about 2.5 to 3 h after first concentrate distribution and just before new silage was distributed.

The chemical composition of the feed ingredients and that of the whole diet consumed by the rumen inoculum donor cows are provided in the Table 7. The sampling of silage (0.5 kg per sample) and concentrates (0.5 kg per sample) was conducted on a weekly basis. The collected samples were stored at -20°C until further analysis. The analysis encompassed dry matter content, crude protein, neutral detergent fibre, total fat, starch, ash, and for silage also D-value and fermentation characteristics. The pH of the silage was analysed on the day of feed sampling.

Table 7 Chemical composition of the feed ingredients and the whole diet consumed by rumen inoculum donor cows.

Nutrients, g/kg of dry matter (DM)	Grass - Red clover silage	Concentrates	Whole diet
DM	306	880	405
Ash	79.4	103	88.3
Crude protein	152	210	174
Total fat	26.7	15.0	22.3
Neutral detergent fibre	553	209	424
Starch	2.30	240	91.5
ME, MJ/kg DM	10.5	11.7	11.0

Fermentation quality of silage (in dry matter): lactic acid 1,56 %; ethanol 0,15%; sugars 2,25%; acetic acid 0,17%; propionic acid 0,10%; NH_3-N 0,04% of total N; pH 4,21.

4.4 Protocol for incubations

Adequate amount of artificial saliva was prepared, and pH adjusted to 6.8 with carbon dioxide in accordance with McDougall (1948). This buffer was placed to the heating cabinet (40°C) the day before the incubation. In advance, 3.78 g of experimental feed DM were weighted into incubation bottles in accordance with experimental design (Table 2).

On the incubation day, the incubation bottles were fixed in incubator with motors (Figure 1 and 2) and 133 ml of buffer was dispensed into each of them. The bottles were sealed and flushed with N_2 to obtain anaerobiosis. Then agitation included 5 minutes of mixing followed by 10-minute intervals of no mixing, with heating at 39°C turned on. The mixing speed was set at 60% to ensure effective media mixing throughout the incubation process.

Simultaneously, at the barn, solid and liquid rumen contents were collected 2.5-3 h post morning concentrate feeding from three different locations (front, middle, and rear) within the rumen of three fistulated cows. The fluid from the rumen was collected using ruminal extraction pump (Biltema, Helsinki, Finland) and metal sampling parts (Alimetrics Oy., Espoo, Finland) at a volume of 1500 ml from each cow, with 500 ml collected from each rumen location. The collected fluid was then transferred into pre-warmed thermosbottles of 1500 ml. Additionally, approximately 10 ml of rumen fluid from each cow was collected in a plastic bottle and placed in ice for later pH analysis in the laboratory. In addition to fluid, solid rumen digesta was extracted by hand from

each cow, taking approximately two fists from each rumen location into 10 L bucket. The digesta was quickly mixed and solid material extracted by squeezing two fists of solid material through one layer of cheesecloth. The extracted solid digesta was then placed into bottles containing 900 ml of buffer, mixed thoroughly, and placed in a Styrofoam box filled with warm water at around 39°C. All the collected samples were promptly transported to the laboratory.

In the laboratory, the rumen fluid of each cow was filtered through two layers of sieves (1.5 and 0.25 mm) with each cow contributing approximately 860 ml of filtered rumen fluid. Subsequently, the contents of solid rumen digesta bottle was squeezed through cheesecloth and filtered using the same two layers of sieves to obtain 860 ml of buffer with solid-adherent microbes from each cow. This process allowed the solid and liquid rumen microbes from all three cows to be pooled together in equal volumes. The filtration process took place in a water bath with a temperature of approximately 39°C, with continuous flushing with CO_2 at a flow rate of 6 L/min. After filtration, the resulting combined rumen inoculum was placed in a heating cabinet at a temperature of 40°C for 15 minutes. Next, 266 ml of the mixed rumen inoculum was dispensed into each of the 15 incubation bottles and flushed with N_2 to obtain anaerobiosis. All the bottles in the water bath were sealed and connected to the flow cells unit (FCU) of the gas volume measuring device (Figure 1 and 2), following the instructions provided in the Gas Endeavour® manual. Subsequently, the *in vitro* system was started, and the fermentation process was run and monitored via Gas Endeavour® software for a duration of 24 hours.

4.5 Experimental measurements

4.5.1 Feeds chemical analysis and energy calculations.

All feeds utilized in this study were analyzed at the Animal Science laboratory of the University of Helsinki for dry matter, crude protein, total fat, NDF, starch and ash. To determine dry matter content feed samples were dried in an oven (Memmert, Memmert GmbH, Schwabach, Germany) at 105°C for 20-24 hours until the constant weight was achieved. To determine silage´s dry matter content the correction for volatiles according to Huida et al. (1986) was used. Prior to secondary dry matter analysis, feed samples were dried in a Memmert ventilating drying cabinet (Memmert GmbH, Germany) initially at 103°C for an hour, followed by two days of drying at 50°C. The dried samples were ground using a 0,8-1 mm sieve and a hammer mill (septic mill

KT-3100, Koneteollisuus Oy, Finland). Secondary dry matter was determined by subjecting the dried and ground samples to oven (Memmert, Memmert GmbH, Schwabach, Germany) at temperature of 103 ºC for a duration of 16 hours.

Ash was determined by incinerating the samples in a muffle furnace (Heraeus Thermicon T, Heraeus, Hanau, Germany) at a temperature of 600ºC for a period of 20 to 24 hours. The analysis of crude protein was conducted using the Kjeldahl method, following the AOAC 1995 standard. The equipment used included the Tecator combustion device (Tecator Digestion Auto and Tecator Scrubber) for digestion, as well as distillation and titration equipment (FOSS Kjeltec Auto 2300, Foss, Hillerød, Denmark) for further processing and measurement. The neutral detergent fibre (NDF) was analysed with the method described by Van Soest et al. (1991) with sodium sulphite application. In addition, the NDF content of starch containing feeds oats, maize silage, and faba bean silage was determined with α-amylase. The NDF concentrations were reported without residual ash. The FiberTherm FT12 automatic extractor (Gerhardt, Königswinter, Germany) was used for the analysis. Starch was determined according to AOAC method 996.11. The device used for colorimetric method was Shimadzu UV-VIS mini 1240 (Shimadzu Europa GmbH, Duisburg, Germany). For starch, the amyloglucosidase/α-amylase method was employed using Megazyme's K-TSTA kit (Megazyme, Co. Wicklow, Ireland). Manufacturer's protocol version c was applied, preceded by ethanol washing (points 1 to 5 of Guide e). The determination of total fat content was carried out through petroleum ether extraction after HCl hydrolysis (FOSS Soxtec 8000 extraction unit, SoxCap 2047 hydrolysis unit, FOSS Analytical, Hillerød, Denmark). Analysis was made according to the manufacturer's instructions.

The digestible organic matter concentration in DM (D-value) for silage was determined with the methodology outlined by Friedel (1990) with the variation introduced by Nousiainen et al. (2003). The analysis involved assessing the *in vitro* digestibility of the organic matter in the silages using the pepsin-cellulase method. The resulting *in vitro* digestibility values were subsequently converted to *in vivo* digestibility by employing the correction equations developed by Huhtanen et al. (2006). The concentration of metabolizable energy in forages and concentrates were calculated according to the Finnish feed evaluation system (Luke 2023). Calculations for forages are based on D-value using the following equations - silages ME (MJ) = 0.016 × D-

value (g/kg DM). In case of concentrates the metabolizable energy was calculated based on the amount of digestible nutrients in the feed using the following equation:

Equation 1 Metabolizable energy for concentrate feeds (Luke 2023)

$$(\mathrm{MJ/kg\ DM}) = (15.2 \times \mathrm{dCP} + 34.2 \times \mathrm{dCFat} + 12.8 \times \mathrm{dCF} + 15.9 \times \mathrm{dNFE})/1000 \quad (1)$$

Where:
dCP = digestible crude protein, g/kg DM
dCFat = digestible crude fat, g/kg DM
dCF = digestible crude fibre, g/kg DM
dNFE = digestible nitrogen free extract, g/kg DM.

For the digestibility of concentrate nutrients values from Finnish Feed Tables were used (Luke 2023). In order to calculate NFE (nitrogen free extract, g/kg DM) for concentrate feeds the following equation was:

Equation 2 Nitrogen free extract for concentrate feeds (Luke 2023)

$$\mathrm{NFE} = 1000 - (\mathrm{CP} + \mathrm{CF} + \mathrm{EE} + \mathrm{Ash}) \quad (2)$$

Where:
CP = Crude Protein, g/kg DM
CF = Crude Fiber, g/kg DM
EE = Ether Extract (Crude Fat), g/kg DM

4.5.2 Rumen fermentation

After 24 hours of incubation, Gas Endeavour® device was turned off according to the manual and incubation bottles were immediately placed on ice and pH was recorded (SevenCompactTM S220, Mettler-Toledo Ltd, Leicester, UK). For VFA analysis, 1 ml of the fermented fluid was collected from each vial into Eppendorf tubes, frozen and stored at -20 °C until analyzed. The VFA was analysed with liquid chromatograph (Waters Acquity UPLC, Waters, Milford, MA, USA), Waters' column 186004097 (Waters MassTrak AAA, Waters) and UV detector in accordance with method described by Lamminen et al. (2017).

4.5.3 Dry matter digestibility

After VFA sampling and pH measurements, each incubation bottle was cleaned onto a designated glass plate using Elix water to ensure safe transfer of all feed particles.

The samples were then placed in an oven at 103°C for 24 hours. After weighing the samples, the DM digestibility was calculated according to the following equation:

Equation 3 Dry matter digestibility

$$\text{DM digestibility (\%)} = \frac{(\text{Feed DM into bottle}) - (\text{Feed DM left in bottle})}{\text{Feed DM into bottle}} * 100 \quad (3)$$

Where:

- DM into bottle: Feed DM weighed into the bottle (in grams).
- Feed DM left in the bottle: Weight of the DM in the feed sample left in the bottle after incubation (in grams).

The formula for calculating DM digestibility involves determining the feed DM left in the bottle that is obtained by subtracting the weight of the empty drying plate and the weight of blind bottle contents from the weight of the treatment plate after drying.

4.5.4 Total gas and methane production

The Gas Endeavour® system utilized gas normalization to standardize gas concentrations to standard conditions (STP), encompassing a temperature of 0°C, atmospheric pressure of 1 atm, and zero moisture content, thereby improving the precision of gas production measurements. The standard temperature and pressure (STP) as well as mixing time (on and off) were consistent across all incubation trials in this study. To eliminate over/underestimation, specific adjustments were applied for methane monitoring. These adjustments included a reactor headspace volume of 295 ml, an initial headspace removable gas concentration of 0%, and a final headspace removable gas concentration of 70%. These refinements further improved the precision and reliability of gas measurements, particularly for methane production, within the Gas Endeavour® system. Gas production data, including total gas and methane, were automatically recorded at 15-minute intervals throughout the 24-hour incubation period, providing a comprehensive and continuous profile of the fermentation process.

4.6 Statistical Analysis

Statistical analysis was conducted using analysis of variance and SAS software version 9.4 (Statistical Analysis Systems Institute Inc., Cary, NC, USA). At 24 h incubation endpoint, the data on DM digestibility, gas production per digested dry matter and rumen fermentation patter were analysed using MIXED procedure and the fixed factors considered were run and diet and the random factor was bottle. For cumulative

methane production data and methane production rate a model with repeated measures design was used. The model had run, diet, hour, and hour-diet interaction as fixed effects and bottle as random effect with the Kenward-Roger correction. The AR (1) covariance structure was applied with a bottle within a run as the subject for repeated measures. In case of hour-diet interaction, the timepoints in question were analysed separately with the same model and orthogonal contrast as used for the 24 h endpoint data.

In trial 1, the pre-designed orthogonal contrasts were

1) Biochar Linear (1)
2) Biochar Quadratic (2)
3) Grass versus Red clover + Faba bean + Maize (3)
4) Red clover versus Faba bean + Maize (4)
5) Faba bean versus Maize (5)
6) Interaction 1 x 3
7) Interaction 1 x 4
8) Interaction 1 x 5
9) Interaction 2 x 3
10) Interaction 2 x 4
11) Interaction 2x 5

In trial 2, the pre-designed orthogonal contrasts were

1) Low vs. high forage (1)
2) No inhibitor vs. inhibitor (2)
3) BC vs. PX (3)
4) BC and PX vs BC+PX (4)
5) Interaction 1 x 2
6) Interaction 1 x 3
7) Interaction 1 x 4

Normal distribution assessment of the data was performed using residuals from the statistical model, Shapiro-Wilk test, and visual inspection of distribution and probability plot for residuals. In case of abnormal distribution, outliers, defined as data

points beyond three times the standard deviation from the mean, were systematically removed from the dataset. Consequently, the data displayed a normal distribution pattern across all recorded results. In case of outlier removal, the highest standard error of the mean (SEM) was reported in Tables.

Statistical significance was determined using the P-value, where $p<0.05$ was significant and P-value between 0.05 and 0.10 was regarded as a tendency towards significance.

5 Results

5.1 Potential of increasing biochar levels to reduce ruminal gas production *in vitro* (Pretrial)

The preliminary trial revealed only subtle effects of different levels of biochar inclusion in grass silage-based diet (65% in diet DM). While all biochar dosages showed a numerical reduction in gas production (nml/g digested DM) to some extent, the most promising outcome was evident at biochar level of 0.25% DM (Table 8), whereas higher inclusion rates did not offer additional benefits. However, to be sure that adequate amount was delivered under various dietary conditions, also the subsequent level 0.5% DM was selected. Consequently, a decision was made to proceed with these two specific biochar levels in trials 1 and 2.

Table 8 Effect of different levels of biochar inclusion in the diet on dry matter digestibility and gas production

Biochar in DM,	Digestibility,	Gas production, nml/ g digested DM			Decrease relative to 0 BC, %		
%	%	CH_4	CO_2	Total	CH_4	CO_2	Total
0 BC	35,0	131	311	442			
0.1 BC	37,0	119	151	370	9	51	16
0.25 BC	38,3	114	127	341	13	59	23
0.5 BC	34,5	124	289	413	5	7	7
1 BC	34,1	124	290	414	5	7	6
2 BC	36,7	114	236	350	13	24	21

DM, dry matter; BC, biochar

5.2 Effect of biochar on feed digestibility and ruminal gas production with different basal forage options *in vitro* (Trial 1)

5.2.1 Effect on dry matter digestibility

Biochar did not significantly affect the digestibility of dry matter within different diet treatments (Table 9). However, diets containing maize silage had slightly higher digestibility than those containing faba bean silage ($p=0.06$).

5.2.2 Effect on rumen fermentation

Biochar had no significant effect on the final rumen pH across different dietary treatments. Nevertheless, the final rumen pH was lower in maize compared to faba bean silage containing diets ($p<0.001$; Table 10). The effects of biochar and silage plant species on rumen fermentation were negligible (Table 10). With grass silage treatment, biochar slightly reduced the rumen fluid VFA content ($p=0.003$) and the molar

proportion of acetic acid in VFA (p= 0.075). However, these effects were not observed when the feeding regimen involved silage mixtures (interaction 1 x 3; Table 10). Furthermore, the treatment did not affect the molar proportion of propionic acid in the VFA (Table 10).

5.2.3 Effect on rumen gas production

The biochar and silage plant species had relatively minor impacts on rumen gas production, either methane or carbon dioxide (Table 9). Overall, methane production was lower with grass silage ($p = 0.032$) compared to silage mixtures (Table 9). However, when considering methane production per digested DM, this difference disappeared. Maize silage treatment increased the total amount of methane and carbon dioxide formed in the rumen compared to faba bean treatment ($p<0.001$). But upon considering gas production per digestible DM, the difference was no longer evident (Table 9).

There was diet-time interaction throughout the 24-hour incubation period ($p<0.001$) in cumulative methane production across treatments. Notably, grass silage treatment consistently exhibited lower cumulative methane production compared to the silage mixtures (Figure 4). Particularly within the initial 12 hours, this distinction was highly significant ($p\leq0.001$), gradually diminishing ($p<0.01$ for 13-18 hours, after that $p<0.05$) towards the end of incubation period.

Table 9 Effect of forage species and biochar inclusion in the diet on dry matter digestibility and gas production in vitro (Trial 1)

Silage treatment	Biochar in DM, %	DM digestibility, %	Gas production, nml			Gas production, nml/g digested DM		
			CH_4	CO_2	Total	CH_4	CO_2	Total
Grass (G)	0	29.8	147	399	547	132	357	489
Grass	0.25	28.8	146	402	548	135	369	504
Grass	0.50	30.7	147	388	535	127	334	460
Red clover (RC)	0	30.7	148	400	547	130	357	486
Red clover	0.25	30.2	146	402	547	126	348	473
Red clover	0.50	30.2	152	412	563	133	361	494
Faba bean (FB)	0	29.0	144	385	533	137	359	495
Faba bean	0.25	28.0	146	385	531	140	370	510
Faba bean	0.50	29.2	146*	364	503	128	336	463
Maize (M)	0	31.5	159*	429	580	128	364	493
Maize	0.25	30.0	155	429	582	136	379	515
Maize	0.50	29.9*	159	435	593	136*	375*	512*
SEM		1.14	3.55	14.6	18.1	7.3	21.3	25.2
Contrast								
Biochar linear (1)		0.724	0.398	0.656	0.743	0.874	0.508	0.583
Biochar quadratic (2)		0.139	0.194	0.674	0.809	0.414	0.249	0.275
Grass vs. RC+FB+M (3)		0.868	0.032	0.233	0.272	0.750	0.444	0.508
RC vs. FB+M (4)		0.276	0.137	0.961	0.901	0.273	0.450	0.376
FB vs. M (5)		0.051	<0.001	<0.001	<0.001	0.801	0.212	0.386
Interaction 1 x 3		0.326	0.478	0.592	0.608	0.494	0.440	0.438
Interaction 1 x 4		0.926	0.499	0.290	0.313	0.803	0.702	0.711
Interaction 1 x 5		0.375	0.866	0.178	0.115	0.160	0.306	0.252
Interaction 2 x 3		0.567	0.774	0.632	0.718	0.630	0.460	0.493
Interaction 2 x 4		0.603	0.589	0.602	0.525	0.205	0.253	0.226
Interaction 2 x 5		0.830	0.277	0.474	0.467	0.759	0.618	0.635

*One observation discarded as outlier; DM, dry matter; G, grass silage; RC, red clover-grass silage mixture; FB, faba bean-grass silage mixture; M, maize-grass silage mixture.

Table 10 Effect of forage species and biochar inclusion in the diet on rumen fermentation in vitro (Trial 1) *

Silage treatment	Biochar in DM, %	pH	Total VFA, mmol/l	Molar proportions, mmol/mol						
				Acetate	Propionate	Butyrate	Isobutyrate	Isovalerate	Valerate	Caproate
Grass (G)	0	6.55	110	622	179	133	10.9	9.72	19.1	18.0
Grass	0.25	6.55	106	618	180	135	10.9	9.43	18.8	19.2
Grass	0.50	6.55	106	619	181	134	11.1	9.67	19.3	18.5
Red clover (RC)	0	6.57	106	619	180	134	10.8	9.20	19.3	19.0
Red clover	0.25	6.56	106	618	180	135	10.7	9.24	19.1	19.1
Red clover	0.50	6.56	107	620	181	132	11.0	9.81	19.1	18.9
Faba bean (FB)	0	6.57	107	619	180	134	10.8	9.33	19.3	19.5
Faba bean	0.25	6.56	106	618	182	135	10.7	9.26	18.8	18.9
Faba bean	0.50	6.57	108	619	179	134	11.0	9.76	19.2	19.3
Maize (M)	0	6.50	106	622	181	130	11.2	9.88	19.4	18.1
Maize	0.25	6.51	106	621	180	134	10.7	9.24	19.2	18.6
Maize	0.50	6.51	108	620	180	136	10.4	8.99	18.6	18.4
SEM		0.008	1.2	1.1	0.9	1.7	0.22	0.242	0.35	0.54
Contrast										
Biochar linear (1)		0.555	0.963	0.348	0.692	0.340	0.901	0.888	0.440	0.791
Biochar quadratic (2)		0.677	0.068	0.097	0.460	0.195	0.293	0.084	0.287	0.492
Grass vs. RC+FB+M (3)		0.463	0.473	0.965	0.506	0.893	0.408	0.216	0.877	0.449
RC vs. FB+M (4)		<0.001	0.755	0.575	0.958	0.897	0.766	0.987	0.619	0.603
FB vs. M (5)		<0.001	0.811	0.027	0.971	0.589	0.650	0.684	0.841	0.057
Interaction 1 x 3		0.606	0.003	0.075	0.154	0.960	0.428	0.798	0.314	0.584
Interaction 1 x 4		0.712	0.934	0.392	0.107	0.056	0.209	0.046	0.533	0.870
Interaction 1 x 5		0.738	0.919	0.403	0.638	0.103	0.031	0.008	0.295	0.650
Interaction 2 x 3		0.694	0.695	0.504	0.990	0.888	0.829	0.952	0.586	0.204
Interaction 2 x 4		0.538	0.471	0.754	0.233	0.553	0.987	0.957	0.999	0.848
Interaction 2 x 5		0.378	0.687	0.633	0.141	0.792	0.755	0.824	0.269	0.419

DM, dry matter; VFA, volatile fatty acids; G, grass silage; RC, red clover-grass silage mixture; FB, faba bean-grass silage mixture; M, maize-grass silage mixture;

*Samples for analysis were taken at the end of 24 hours incubation period

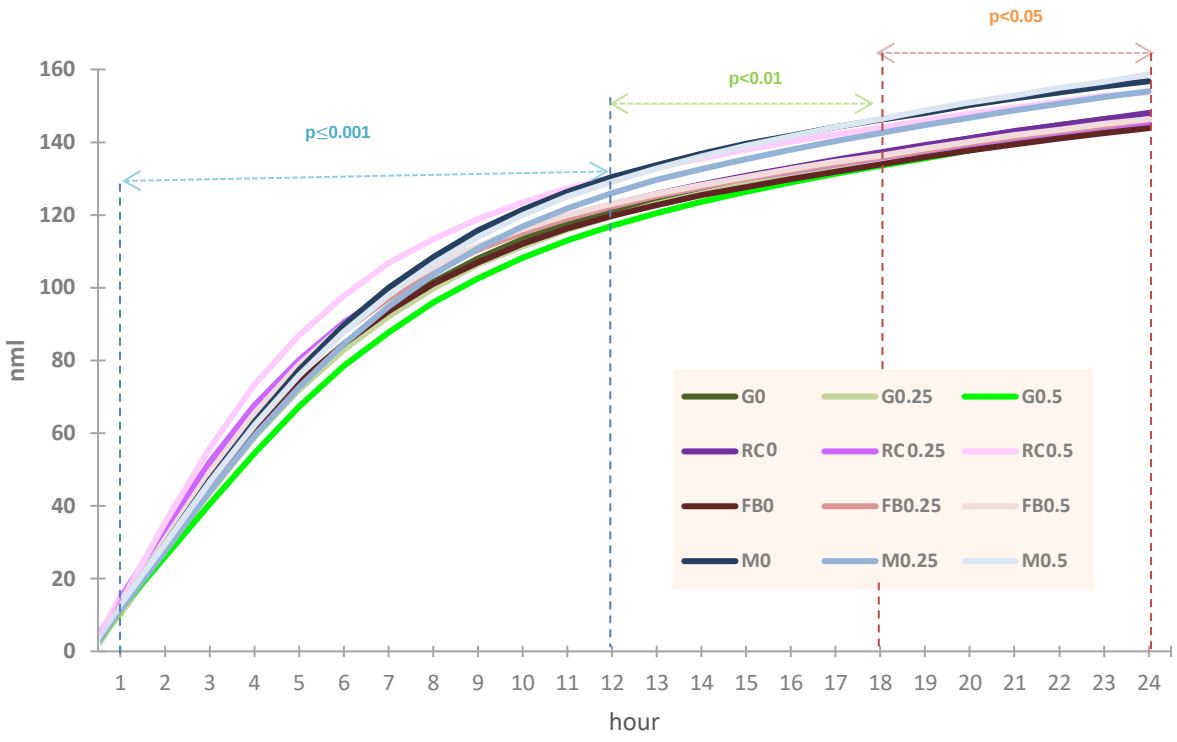

Figure 4 Cumulative methane production during 24 hours incubation in vitro. Contrast grass silage versus other silage mixtures. G0 - grass silage with no biochar; G0.25 – grass silage with 0.25% biochar; G0.5 - grass silage with 0.5% biochar; RC0 - red clover-grass silage mixture with no biochar; RC0.25 – red clover-grass silage mixture with 0.25% biochar; RC0.5 – red clover-grass silage mixture with 0.5% biochar; FB0 – faba bean-grass silage mixture with no biochar; FB0.25 – faba bean-grass silage mixture with 0.25% biochar; FB0.5 – faba bean-grass silage mixture with 0.5% biochar; M0 – maize-grass silage mixture with no biochar; M0.25 – maize-grass silage mixture with 0.25% biochar and M0.5 – maize-grass silage mixture with 0.5% biochar.

Red clover treatment demonstrated higher cumulative methane production ($p \leq 0.05$) compared to the faba bean and maize treatments during the initial 8 hours of incubation (Figure 5). This difference was highly significant within the first 6 hours of incubation ($p < 0.001$), then gradually decreasing ($p < 0.05$) until it vanished by the 9th hour of incubation. Subsequently, from the 9th hour onward until the end of incubation, a significant ($p \leq 0.05$) disparity in cumulative gas production emerged between faba bean and maize treatments (Figure 6). Faba bean showed lower methane production than maize and this discrepancy showed an escalating trend until the end of incubation period.

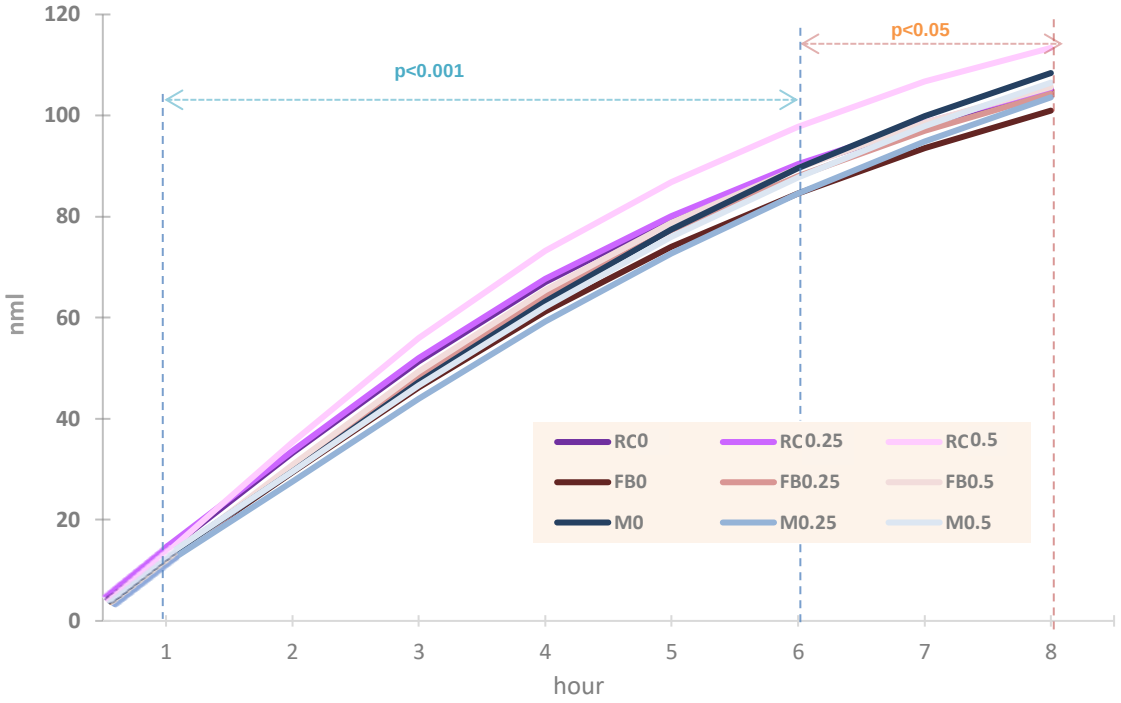

Figure 5 Cumulative methane production, first 8 hours of 24h incubation period in vitro. Contrast red clover treatment versus faba bean and maize treatments. RC0 - red clover-grass silage mixture with no biochar; RC0.25 – red clover-grass silage mixture with 0.25% biochar; RC0.5 – red clover-grass silage mixture with 0.5% biochar; FB0 – faba bean-grass silage mixture with no biochar; FB0.25 – faba bean-grass silage mixture with 0.25% biochar; FB0.5 – faba bean-grass silage mixture with 0.5% biochar; M0 – maize-grass silage mixture with no biochar; M0.25 – maize-grass silage mixture with 0.25% biochar and M0.5 – maize-grass silage mixture with 0.5% biochar.

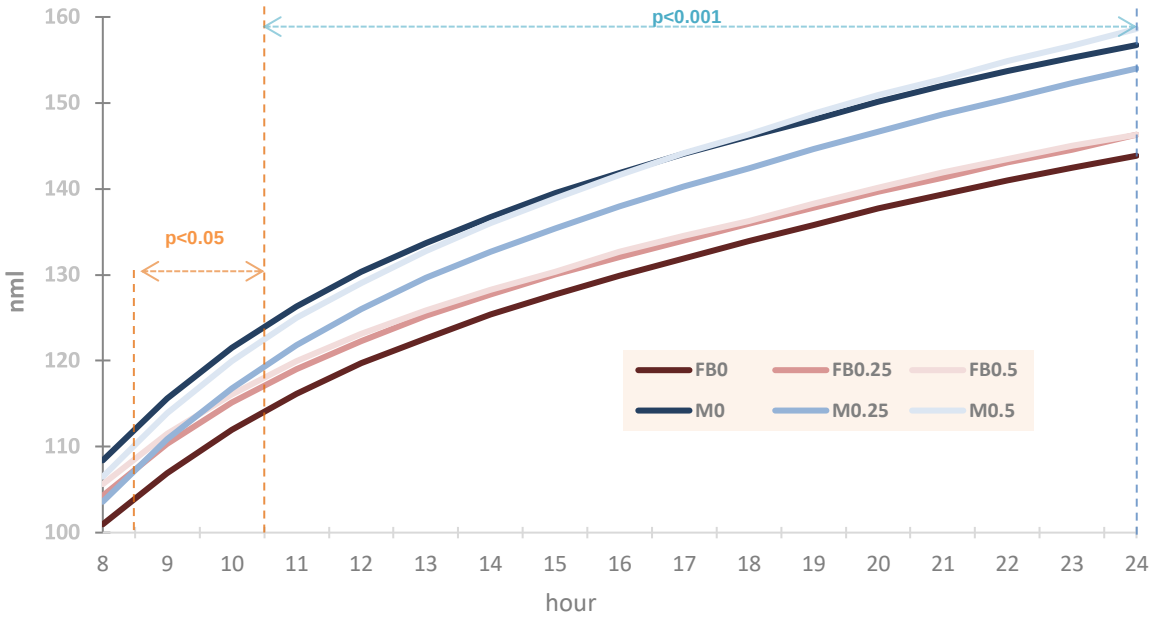

Figure 6 Cumulative methane production, 8-24 hours of 24h incubation period in vitro. Contrast maize treatment versus faba bean treatment. FB0 – faba bean-grass silage mixture with no biochar; FB0.25 – faba bean-grass silage mixture with 0.25% biochar; FB0.5 – faba bean-grass silage mixture with 0.5% biochar; M0 – maize-grass silage mixture with no biochar; M0.25 – maize-grass silage mixture with 0.25% biochar and M0.5 – maize-grass silage mixture with 0.5% biochar.

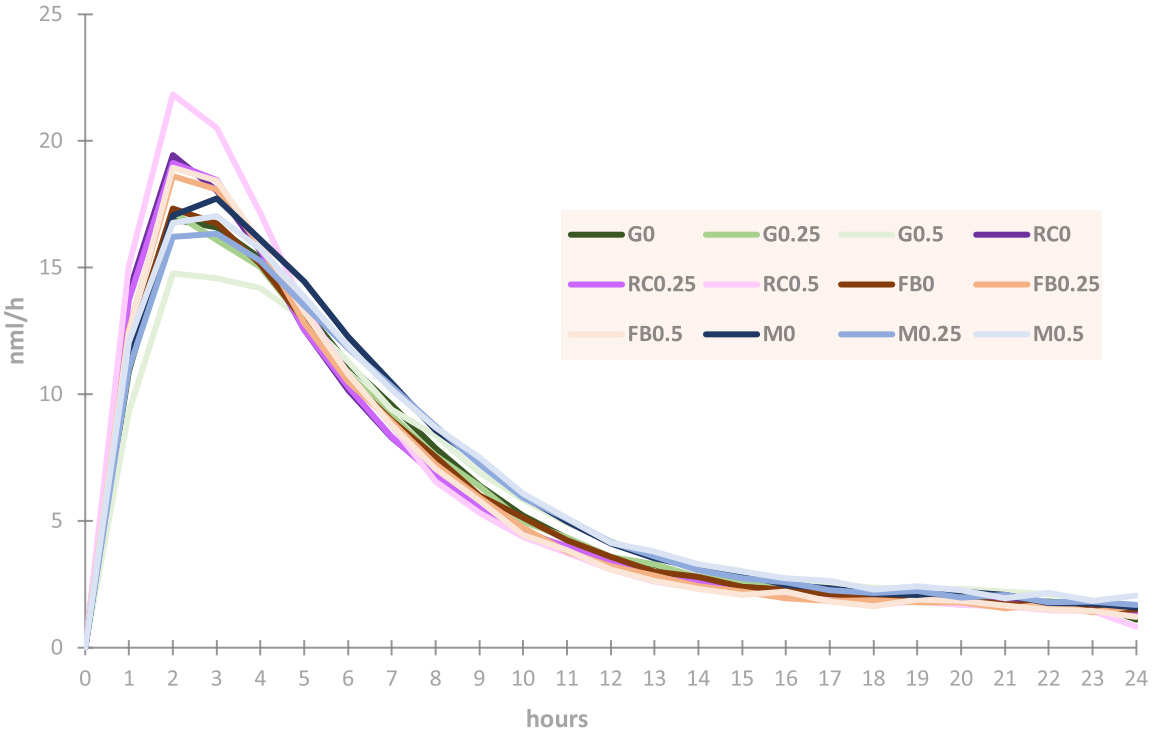

Figure 7 Methane production rate kinetics (nml/h) during the 24 hours incubation period in vitro. G0 - grass silage with no biochar; G0.25 – grass silage with 0.25% biochar; G0.5 - grass silage with 0.5% biochar; RC0 - red clover-grass silage mixture with no biochar; RC0.25 – red clover-grass silage mixture with 0.25% biochar; RC0.5 – red clover-grass silage mixture with 0.5% biochar; FB0 – faba bean-grass silage mixture with no biochar; FB0.25 – faba bean-grass silage mixture with 0.25% biochar; FB0.5 – faba bean-grass silage mixture with 0.5% biochar; M0 – maize-grass silage mixture with no biochar; M0.25 – maize-grass silage mixture with 0.25% biochar and M0.5 – maize-grass silage mixture with 0.5% biochar.

Methane production rate kinetics, calculated as the volume of methane produced within each hour during the incubation period, revealed no significant differences ($p>0.10$) either due to biochar addition or among silage treatments (Figure 7). From the first 2 hours of incubation, a rise in methane production was seen across all treatments, followed by a gradual decline from the 3rd to the 12th hour, and thereafter, methane production reached a plateau, remaining relatively constant until the end of incubation period. The red clover silage treatment with an addition of 0.5% biochar displayed the highest methane production rate during the initial 4 hours. Conversely, the grass silage treatment with 0.5% added biochar demonstrated the lowest methane production rate during the same period; however, these differences were not statistically significant.

5.3 Effect of biochar alone and product X on rumen fermentation at different dietary forage-to-concentrate ratios *in vitro* (Trial 2)

5.3.1 Rumen pH and fermentation

Forage level in the diet had no effect on rumen fluid pH ($p>0.10$, Table 12). However, the presence of product X led to an increased final rumen pH when compared to biochar ($p=0.008$). The use of biochar and/or product X across different forage levels did not result in significant changes in rumen fluid total VFA content ($p>0.10$; Table 12). Forage level in the diet had no major impact on VFA profile. Low F:C ratio had no effect on molar proportion of acetic acid but showed an increase in the molar proportion of butyric acid ($p<0.001$) and a small reduction in the proportions of propionic, isobutyric, isovaleric, valeric, and caproic acids ($p\leq0.011$). Compared to biochar, product X slightly increased the proportion of acetic acid ($p<0.001$) and reduced the molar fraction of propionic acid ($p<0.05$)

5.3.2 Dry matter digestibility and rumen gas production

The addition of biochar or product X, either individually or in combination, did not significantly affect rumen dry matter digestibility across different forage levels ($p>0.05$; Table 11). The potential inhibitors tested had no effect on rumen gas production, either methane or carbon dioxide (Table 11). The combination of product X and biochar within the high forage diet showed the lowest methane, carbon dioxide, and total gas production values compared to other treatments. However, this difference wasn't statistically significant and disappeared when gas production rate was calculated per digested dry matter (Table 11). There was no statistically significant difference also in cumulative methane production between the treatments (Figure 8). However, the lowest cumulative methane production rate was observed with the low forage diet supplemented with product X, while the highest rate occurred when the high forage diet was supplemented with biochar. There was no diet-time interaction ($p > 0.05$; Figure 8)

Table 11 Effect of forage to concentrate ratio and potential methane inhibitors on dry matter digestibility and gas production in vitro (Trial 2)

F:C ratio	Methane inhibitor	DM digestibility, %	Gas production, nml			Gas production, nml/g digested DM		
			CH_4	CO_2	Total	CH_4	CO_2	Total
45:55 (low forage)	-	34.5	147	414	562	111	302	413
45:55 (low forage)	PX	33.5	147*	389	531	110	297	408
45:55 (low forage)	BC	34.5	146*	405	543	118	327	444
45:55 (low forage)	BC+PX	32.1	146	401	547	117	314	432
65:35 (high forage)	-	33.9	147	393	540	115	303	418
65:35 (high forage)	PX	31.8	146	401	547	117	319	436
65:35 (high forage)	BC	35.2	147	402	552	110	286	396
65:35 (high forage)	BC+PX	33.0	145	388	533	114	298	412
SEM		1.50	5.29	22.5	26.1	7.50	22.0	28.9
Contrast								
Low vs. high forage (1)		0.858	0.969	0.315	0.711	0.982	0.514	0.614
No inhibitor vs. inhibitor (2)		0.478	0.840	0.380	0.307	0.747	0.760	0.753
BC vs. PX (3)		0.139	0.946	0.294	0.434	0.968	0.939	0.945
BC and PX vs. BC+PX (4)		0.361	0.744	0.529	0.689	0.726	0.943	0.961
Interaction 1 x 2		0.840	0.976	0.157	0.134	0.640	0.651	0.646
Interaction 1 x 3		0.433	0.856	0.423	0.771	0.303	0.114	0.146
Interaction 1 x 4		0.581	0.849	0.214	0.134	0.829	0.819	0.814

* One observation discarded as outlier; F:C ratio, forage to concentrates ratio; DM, dry matter; PX, product X; BC, biochar

Table 12 Effect of forage to concentrate ratio and potential methane inhibitors on rumen fermentation in vitro (Trial 2)

F:C ratio	Methane inhibitor	pH	Total VFA, mmol/l	Molar proportions, mmol/mol						
				Acetate	Propionate	Butyrate	Isobutyrate	Isovalerate	Valerate	Caproate
45:55 (low forage)	-	6.51	107	614	174	145	11.0	9.63	19.7	19.3
45:55 (low forage)	PX	6.53	107	616	174	143	11.1	9.59	19.6*	19.2
45:55 (low forage)	BC	6.51	108	614	176	143	11.0	9.61	19.7*	19.2
45:55 (low forage)	BC+PX	6.53	109	615	173	145	11.1	9.65	19.7	18.6
65:35 (high forage)	-	6.52	108	613	179	138	11.3	9.89	20.0	21.1
65:35 (high forage)	PX	6.53	108	615	177	137	11.5	10.1	19.8	20.4
65:35 (high forage)	BC	6.52	108	614	179	137	11.4	10.0	19.7	20.7
65:35 (high forage)	BC+PX	6.54	107	616	178	137	11.4	9.87	19.9	20.3
SEM		0.007	1.2	0.5	0.77	0.68	0.08	0.133	0.12	0.26
Contrast										
Low vs. high forage (1)		0.366	0.933	0.670	<0.001	<0.001	<0.001	<0.001	0.011	<0.001
No inhibitor vs. inhibitor (2)		0.045	0.636	<0.001	0.505	0.062	0.233	0.591	0.113	0.022
BC vs. PX (3)		0.008	0.981	<0.001	0.020	0.950	0.344	0.795	0.679	0.673
BC and PX vs BC+PX (4)		0.021	0.699	0.054	0.107	0.284	0.765	0.425	0.437	0.048
Interaction 1 x 2		0.755	0.469	0.644	0.346	0.431	0.707	0.475	0.437	0.471
Interaction 1 x 3		0.685	0.629	0.897	0.962	0.828	0.773	0.683	0.366	0.584
Interaction 1 x 4		0.745	0.474	0.605	0.150	0.048	0.509	0.167	0.580	0.418

* One observation discarded as outlier; F:C ratio, forage:concentrates ratio; VFA, volatile fatty acids; PX, product X; BC, biochar

Samples for analysis were taken at the end of 24 hours incubation period.

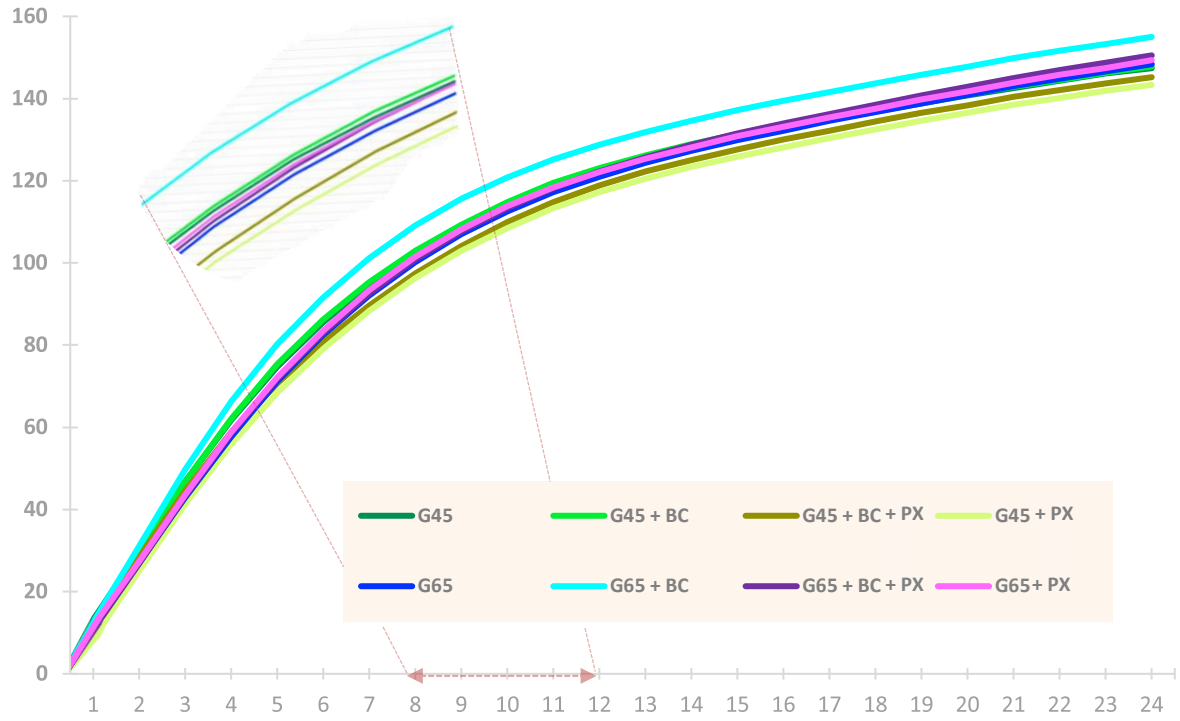

Figure 8 Cumulative methane production, 24 hours incubation period in vitro. Cross-sectional graph for 8-12h incubation period G45, 45 % of grass silage in diet DM; G45 + BC, 45 % of grass silage in diet DM supplemented with 0.5% biochar; G45 + BC + PX, 45 % of grass silage in diet DM supplemented with 0.5% biochar and 1.35% of product X; G45 +PX, 45 % of grass silage in diet DM supplemented with 1.35% of product X; G65, 65 % of grass silage in diet DM; G65 + BC, 65 % of grass silage in diet DM supplemented with 0.5% biochar; G65 + BC + PX, 65 % of grass silage in diet DM supplemented with 0.5% biochar and 1.35% of product X; G65 + PX, 65 % of grass silage in diet DM supplemented with 1.35% of product X.

Methane production rate kinetics revealed no significant differences either due to addition of methane inhibitors (biochar and/or product x) or across forage levels ($p>0.05$, Figure 9). From the first 2 hours of incubation, a rise in methane production was seen across treatments, followed by a gradual decline from the 3rd to the 12th hour, and thereafter, methane production reached a plateau, remaining relatively constant until the end of incubation period. The high forage diet with biochar showed the highest methane production rate during the initial 5 hours, however these observations were not statistically significant.

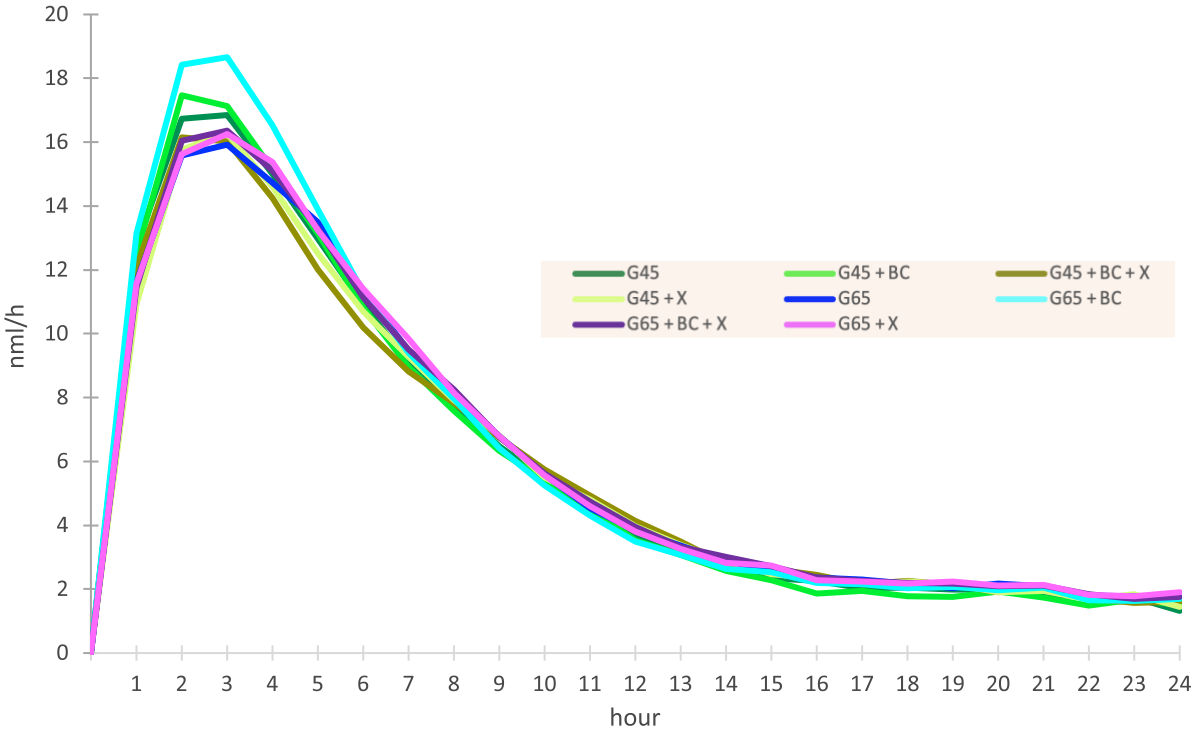

Figure 9 Methane production rate kinetics (nml/h) during the 24 hours incubation period in vitro. G45, 45 % of grass silage in diet DM;G45 + BC, 45 % of grass silage in diet DM supplemented with 0.5% biochar; G45 + BC + X, 45 % of grass silage in diet DM supplemented with 0.5% biochar and 1.35% of product X; G45 + X, 45 % of grass silage in diet DM supplemented with 1.35% of product X; G65, 65 % of grass silage in diet DM; G65 + BC, 65 % of grass silage in diet DM supplemented with 0.5% biochar; G65 + BC + X, 65 % of grass silage in diet DM supplemented with 0.5% biochar and 1.35% of product X; G65 + X, 65 % of grass silage in diet DM supplemented with 1.35% of product X.

6 Discussion

6.1 Composition of the feeds

While our study utilized biochar sourced from spruce wood (*Picea abies*), most of previous *in vitro* and *in vivo* studies used biochar sourced from rice husk *Oryza sativa* (Qomariyah et al.2023; Dung et al. 2022; Cabeeza et al.2018; Leng et al. 2012). However, recent work by Benchaar et al. (2023) studied biochar sourced from spruce (*Picea abies*) and black spruce (*Picea mariana*) shavings along with 5 other biochar sources. The pyrolysis temperature range for biochar production is varying a lot within previous studies, starting from 450-500°C (Benchaar et al. 2023) to 900-1000°C (Leng et al. 2012). Current study used biochar pyrolised at 650-700°C, similar temperature range was applied for biochar also in some other studies (Dung et al.2022; Cabeza et al.2018; Table 1). Physical properties of biochar rage also a lot depending on biochar source. For instance, specific surface area of our biochar is 398.6 m^2/g which is similar to spruce biochars (240-266 m^2/g) in Benchaar et al (2023) study and differ drastically for rice husk (20-42 m^2/g) in Cabeeza et al. (2018) study.

The chemical compositions of the forages used in current study were comparable to previous studies (Table 13). In line with previous studies grass silage had higher NDF content, whereas the CP content was lower than that of red clover or faba bean silages but almost double higher than CP content of maize silage (Chowdhury et al. 2023; Tran et al. 2015; Heuze et al. 2015 and Halmemies-Beauchet-Filleau et al. 2014; Table 12). In the current study faba bean silage had slightly lower (- 3g/kg DM) NDF content than red clover and lower (- 40g/kg DM) than maize silage (359 vs. 362 vs 399 g/kg DM respectively), which is not in line with previous studies (Table 13). For instance, NDF content of faba bean silage reported by Heuze et al. 2021 had higher NDF content than red clover silage (+ 56g/kg DM; Heuze et al. 2015) and maize silage (+26 g/kg DM; Heuze et al. 2017). Likewise, Räisänen et al. 2023 reported higher NDF content for faba bean silage (+15g/kg DM) than red clover. The faba bean variety, farm location, ensiling practice and harvesting methods in the current study closely resembled those detailed in the Räisänen et al. (2023) study. Hence, we assume that differences in NDF content could be attributed to climatic conditions during the growing season and the maturity stage at harvest (Frazer et al. 2001; Borreani et al. 2009; Hernandez et al. 2017).

Table 13 Chemical composition of grass, red clover, faba bean and maize silages.

	DM, g/kg	CP g/kg DM	NDF g/kg DM	Starch g/kg DM	D-value g/kg DM
GS - (current study)	253	179	523[a]	-	669
Chowdhury et al. 2023	320	167	510	-	655[c]
Räisänen et al. 2023	284	165	516	-	694
Halmemies-Beauchet-Filleau et al. 2014	236	156	529	10	699
Brask et al. 2013	329	124	515[a]	-	710
Tran at al. 2015	308	139	562	-	-
RCS - (current study)	331	188	362[a]	-	626
Chowdhury et al. 2023	421	178	371	-	627[c]
Räisänen et al. 2023*	320	135	422	10	644
Halmemies-Beauchet-Filleau et al. 2014	233	200	339	16	688
Heuze et al. 2015	277	189	413	-	-
MS - (current study)	359	79	399[a]	278	679
Chowdhury et al. 2023	340	98	380	-	673[c]
Brask et al. 2013	306	93	437[a]	-	880
Heuze et al. 2017	325	69	443	291	-
FBS - (current study)	247	180	359[a]	48	593
Räisänen et al. 2023	278	169	437	31	567
Palmio et al. 2016	203	181	424	9	646
Heuze et al. 2021**	194	178	469	43	-

GS, grass silage; RCS, red clover silage; MS, maize silage; FBS, faba bean silage

DM, dry matter; CP, crude protein; NDF- neutral detergent fiber; NDFom – ash free NDF; OMD, organic matter digestibility

[a]NDFom; [b]Whole-tract apparent digestibility; [c] effective rumen degradability at 8%/h rumen passage rate

*Mixture of red clover (51%) and grass (49%) silages **review for Faba bean (Vicia faba), aerial part, fresh

Grass silage had higher digestibility than red clover silage (D-value 669 vs. 626 g/kg dry matter), which is in line with other studies (Chowdhury et al., 2023, Räisänen et al., 2023). Maize silage was more digestible than faba bean silage (D-value 679 vs. 593 g/kg dry matter) and contained more starch (278 vs. 48 g/kg dry matter). Similar difference in starch content of maize and faba bean silages is in line with previous reports by Heuze et al .2017 and Heuze et al. 2021. Maize silage usually contains higher starch levels compared to other silages, with grass and forage legume silages having minimal starch content (NRC 2001).

In the current study, were investigated typical F:C, including both high (65% grass silage; NDF 453, CP, 179, starch 114 g/kg DM) and low (45% grass silage; NDF 407, CP, 189, starch 179 g/kg DM) F:C ratios. These ratios fall within established ranges: low F:C ratios typically consist of 30-50% forage and 50-70% concentrate, while high F:C ratios usually comprise 50-70% forage and 30-50% concentrate (Van Soest, 1991;

NRC, 2001; McDonald et al., 2011). Moreover, current F:C ratios align with those found in previous studies; for example, Jaakomo et al. (2023) observed low forage diets containing 30% of grass silage and high forage diets containing 70% of grass silage. Additionally, research by Aguerre et al. (2011) investigated the effects of various F:C ratios, on methane emissions in dairy cow diets, further demonstrating the relevance and typicality of our selected F:C ratios in the context of livestock feeding. According to ProAgria report (Huhtamäki 2023), typical F:C ratio for lactating cows in Finland is 55:45. It is noteworthy that the specific ratio within these ranges is subject to variability and depends on factors such as the nutritional requirements of the animals, the production phase, and the desired outcomes (Jaakomo et al., 2023; Chase et al., 2013; McDonald et al., 2011).

6.2 Rumen pH and fermentation pattern

While acetic and butyric acid formation generates hydrogen, propionate synthesis consumes hydrogen. Biochar supplementation potentially redirects this hydrogen from carbohydrate fermentation toward propionate synthesis. This shift limits hydrogen availability necessary for methane production, potentially reducing its synthesis (Qomariyah et al. 2023). In the current study, biochar supplementation did not affect rumen fermentation or alter rumen pH across different silage species (trial 1) or forage levels (trial 2), which contrasts with our initial hypothesis. We anticipated that biochar would alter rumen fermentation, leading to an increase in the molar proportion of propionate. However, the results indicated no significant effect on propionate levels. This outcome is consistent with some previous *in vitro* studies by Tamayao et al. (2021), Dung et al. (2022), Benchaar et al. (2023), and *in vivo* studies by Leng et al. (2012), which also reported no significant impact of biochar supplementation on rumen pH and fermentation.

Interestingly, in treatments involving grass silage (trial 1), biochar slightly reduced the content of rumen fluid volatile fatty acids (VFAs) and the molar proportion of acetic acid in VFAs. This observation aligns with the findings of Qomariyah et al. (2023), who reported a decrease in total VFA and the molar proportion of acetic acid in VFAs in *in vitro* studies with biochar supplementation, following a quadratic pattern. This finding may suggest that a lesser amount of acetate can generate less hydrogen available for methane formation in the rumen, which can suppress methane emission.

Neither biochar nor product X significantly affected the final rumen pH or the total VFA content, diverging from the hypothesis that these additives would exhibit distinct mechanisms in modulating rumen fermentation.

In the context of the effect of plant species alone on rumen pH and fermentation, the current study demonstrated that maize silage exhibited a lower incubation final pH but a higher molar proportion of acetate compared to faba bean. This finding aligns with the results of the study by Chowdhury et al. (2023), where corn silage similarly exhibited a lower fermentation pH but a higher proportion of acetate compared to alfalfa silage. The protein content in legumes (such as alfalfa and faba bean) plays a role in buffering the rumen pH (Broderick, 1995). Legumes typically have a higher protein content compared to grasses or maize, and this protein can potentially moderate changes in acidity during fermentation (Titterton and Maasdorp, 1997; Elamine et al., 2022). The proteins present in the legumes might help stabilize the rumen pH by buffering against the acids produced during fermentation. The study of Aschenbach et al. (2011) showed that proteins facilitate the absorption of fermentation acids in the rumen, aiding in proton removal and thus stabilizing rumen pH. This buffering capacity could be a factor contributing to the observed differences in rumen pH and fermentation outcomes between silages from different plant species.

Higher dietary content of fiber increases the production of acetate, whereas diets with higher content of starch favor propionate formation (Valadares et al., 1999). In present study, despite higher amount of starch in low forage diet (+ 65 g/kg DM) it showed small reduction in molar proportion of propionic acid and no effect on molar proportion of acetic acid. This finding aligns with Dann et al. (2014), who also reported no significant effects of varying starch levels on the acetate-to-propionate ratio in dairy cow diets, suggesting that dietary starch variations might not significantly alter rumen fermentation patterns on all occasions. Moreover, Shingfield et al. (2002) demonstrated that rumen fermentation on grass silage-based diets could be relatively resistant to dietary manipulations, which further supports the notion of the rumen ecosystem's inherent stability against changes in diet composition. However, it is not always the case. Bayat et al. (2017) demonstrated that specific interventions, such as adding sunflower oil to diets, can significantly influence rumen fermentation and methane emissions, indicating the rumen's adaptability under specific conditions. This

suggests that while variations in starch or fiber might not always alter rumen fermentation patterns, precise dietary supplements can effectively induce changes.

Dann et al. (2014) studied the effects of different starch levels in dairy cow diets and found no significant effect on the acetate-to-propionate ratio. This research indicates that variations in dietary starch content may not always lead to significant changes in rumen fermentation patterns (Dann et al., 2014).

6.3 Dry matter digestibility

Nutrient digestibility is important in the context of methane production in ruminants because effective digestion minimizes the substrates available for methanogenic microbes. Efficient nutrient absorption, especially of fibrous feeds, reduces the amount of undigested material entering the hindgut, where some methane is also produced. Improved digestion can lead to changes in rumen fermentation, potentially lowering ruminal methane emissions. For instance, Janssen (2010) notes that enhancing the digestibility of a diet can decrease the methane yield per unit of digested material, making nutrient digestibility a key factor in reducing enteric methane emissions from ruminants.

The findings of current study showed that biochar did not significantly impact dry matter digestibility within different forage species or forage levels, aligning with the hypothesis that biochar would not affect dry matter digestibility. The lack of a significant effect of biochar on dry matter digestibility across different forage species and levels is consistent with prior research that also noted no changes in digestibility due to biochar inclusion. For instance, Tamayao et al. (2021) investigated the impact of three distinct pine-based biochars (F:C 60:40) over 24 h incubation and found no effect on dry matter digestibility, irrespective of the type or dosage/inclusion rate of the biochar up to 20% of DM. Similarly, Benchaar et al. (2023), who studied seven different types of biochar at varying inclusion rates (1%, 2%, and 5% of DM) also over 24 h of incubation (F:C 50:50), reported no alterations in dry matter digestibility, regardless of the biochar type or inclusion rate. Contradictory, study by Saleem et al. (2018) investigating the effects of pine biochar showed a linear increase in nutrient disappearance when biochar was added at 0.5%, 1.0%, and 2.0% of the diet DM to a barley silage-based diet (F:C 60:40). Furthermore, a study by Dung et al. (2022) demonstrated that the inclusion level of biochar during a 48-hour incubation period with F:C 70:30 influenced digestibility outcomes. Dung et al. (2022) observed that

while 2% and 3% rice husk biochar did not affect digestibility, higher levels of 5% and 7% significantly decreased digestibility compared to the diet without biochar. Interestingly, meta-analysis of 17 *in vivo* studies conducted by Qomariyah et al. 2023 showed that while biochar supplementation did not decrease methane emissions in ruminants, it increased nutrient digestibility (dry matter, organic matter, crude protein, NDF).

The variance in *in vitro* outcomes studies suggests that the impact of biochar on digestibility might be contingent upon the dosage or concentration levels used in the diet. Moreover, contradictions in the biochar effect on digestibility between *in vitro* (24h incubation vs. 48h incubation) and *in vivo* studies can imply that microbial adaptation to new feed additives, such as biochar, might require sufficient time and could influence the rumen microbial community and fermentation dynamics. *In vivo* studies typically involve a prolonged exposure of ruminants to biochar in their diet, allowing the rumen microbiota time to adapt to the presence of biochar. This adaptation could lead to changes in microbial efficiency and fermentative processes, potentially enhancing nutrient digestibility and altering methane production. Therefore, it is important to note that the relatively short duration of the *in vitro* incubation period in the current study might not have allowed sufficient time for rumen microorganisms to fully adapt to the presence of biochar, potentially explaining the absence of significant changes in digestibility and methane production.

In addition, current study observed that maize silage mixtures exhibited slightly higher DM digestibility compared to faba bean silage. This finding is consistent with the research conducted by Guevara-Oquendo et al. (2021), who reported a linear decrease in starch digestibility with increasing supplementation of faba bean silage, indicating a comparatively lower digestibility rate than that of maize silage. This pattern aligns with the generally observed higher starch digestibility in maize silage. Furthermore, due to its lower fiber content, maize silage typically results in higher ruminal organic matter digestibility compared to grass silage, thereby influencing the overall digestibility and methane emissions from dairy cows, as demonstrated by Brask et al. (2013).

6.4 Effects on ruminal methane production

6.4.1 Biochar

The current study found that spruce biochar (pH 9.5, pyrolysis temperature 650-700°C), when used across different silage plant species and varying forage-to-concentrate (F:C) ratios, had a minor or no impact on rumen gas production, including both methane and carbon dioxide. Consequently, the hypothesis suggesting a decrease in methane emissions with increasing concentrations of biochar was not confirmed. Dung et al. (2022) observed a linear decrease in methane production *in vitro* using rice husk biochar at levels ranging from 1% to 7%. However, this pattern was not consistently seen in other *in vitro* studies. For example, Benchaar et al. (2023) assessed the impact of spruce biochar at concentrations of 1%, 2%, and 5% and found neither linear nor quadratic effect on methane production. Pereira et al. (2014) also reported no significant impact on methane production with pine biochar at levels of 0.5%, 1%, 2%, and 5%. Furthermore, study by Saleem et al. (2018), which investigated pine biochar inclusions of 0.5%, 1%, and 2%, suggested that biochar's effect on methane production might follow a quadratic rather than a linear trajectory. In the realm of *in vivo* studies, Winders et al. (2019) analysed the effects of 0.8% and 3% pine biochar on six crossbreed steers and also noted a tendency towards a quadratic relationship between biochar concentration and methane mitigation. These findings alongside with the result of current study might indicate that the impact of biochar on reducing methane emissions does not follow a consistent linear model across various concentrations and study conditions, suggesting a quadratic relationship may be more probable.

Methane production rate kinetics, calculated as the volume of methane produced within each hour during the incubation period, revealed no significant differences either due to biochar among silage treatments and F:C ratios. An initial increase in methane production rate during the first 2 hours, followed by a decline and eventual plateau, reflects the typical phases of microbial activity in the rumen upon feed arrival. Initially, methanogens rapidly utilize available substrates, leading to increased methane production. As these readily fermentable substrates diminish, methane production decreases, stabilizing once a balance between substrate availability and microbial activity is achieved. This pattern is consistent with the understanding of rumen fermentation kinetics described in Janssen (2010), which emphasizes the impact of substrate availability and microbial dynamics on methane emissions.

Overall, biochar's relatively minor impact on rumen gas production might be due to its adsorptive properties, which can affect microbial fermentation processes indirectly (Hansen et al., 2012; Tamayo et al., 2021). Furthermore, biochar's effects on methane production can yield in nuanced insights, as demonstrated by various studies (Table1), including Benchaar et al. (2023), Saleem et al. (2018), Cabeza et al. (2018), and Dung et al. (2022). The observed differences in the anti-methanogenic effect of biochar—whether linear, quadratic, or negligible—underscore the complexity of biochar's impact on rumen fermentation, rumen microbiota and methane emissions and highlight the significance of its source, physicochemical properties such as pH level, pyrolysis temperature, inclusion levels, and the duration of incubation. For instance, Benchaar et al. (2023) explored the effects of similarly sourced biochar (spruce) with an alkaline pH range (7.3-7.7), observing no significant impact on methane production, aligning with the outcomes of the current study (Figure 10).

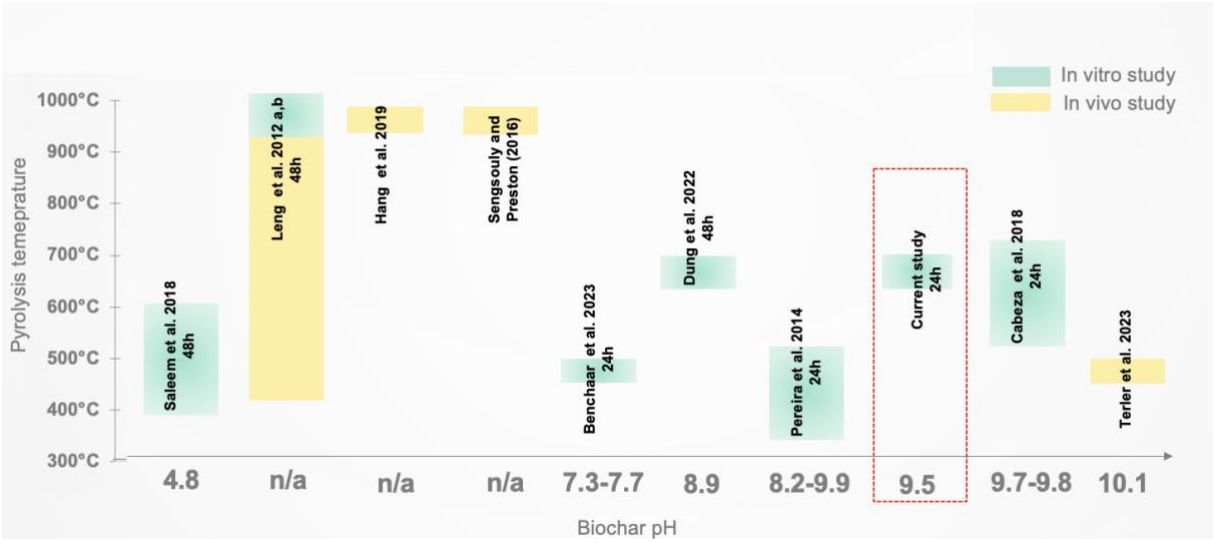

Figure 10 Biochar`s properties in the context of pyrolysis temperature and pH level across *in vitro* and *in vivo* studies

This contrasts with the findings of Saleem et al. (2018), who reported a 25% decrease in methane production using a similarly pine-sourced biochar but with an acidic pH (Figure 11). This variation suggests that the pH level of biochar may play a role in its effectiveness in reducing methane emissions (Dung et al., 2022), indicating a potential area for further research to elucidate the influence of biochar's pH on rumen fermentation processes. Additionally, Cabeza et al. (2018) found that biochar pyrolyzed at 550°C resulted in less methane production, expressed as a ratio of total gas production, compared to biochar pyrolyzed at 700°C. This finding implies that the

pyrolysis temperature of biochar can affect its efficacy in mitigating methane emissions, possibly due to changes in its chemical structure and properties at different temperatures (Qomariyah et al., 2023).

Furthermore, the duration of the incubation period could significantly affect biochar behaviour within rumen fermentation and gas production in the context of microbial dynamic in rumen. Studies conducted over 24-hour incubations showed no significant or minor effect on methane production regardless of the source or dosage (Pereira et al., 2014; Cabeeza et al., 2018; Benchaar et al., 2023). In contrast, studies conducted over 48h of incubation (Hansen et al., 2012; Saleem et al., 2018; Dung et al., 2022) reported a significant reduction (10-25%) in methane production in response to different biochar dosages (Figure 11).

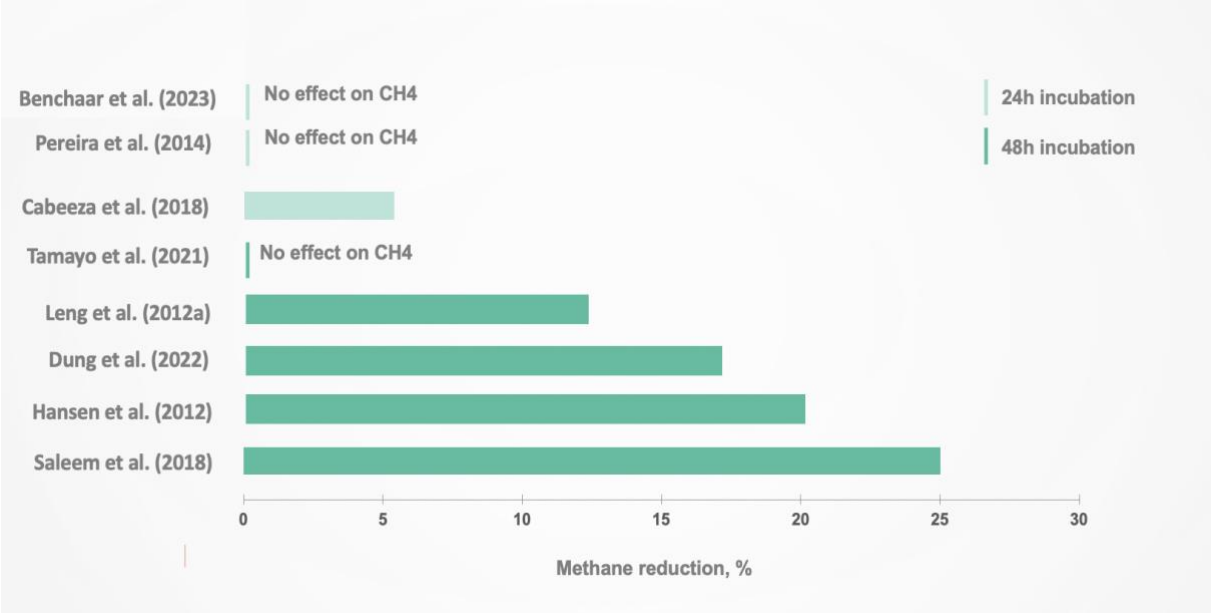

Figure 11 Biochar effect on ruminal methane reduction, comparation of 24h and 48h *in vitro* studies.

Wie et al. (2022), studied the dynamic variation in rumen fermentation over 12 and 24-hour intervals, revealed that the duration of fermentation impacts gas production, concentrations of VFAs, and the degradation rates of structural/nonstructural carbohydrates. Longer incubation times in batch cultures, such as 48 hours, and in particular continuous fermenters of several days or weeks might provide a more comprehensive view of biochar's impact on microbial communities and fermentation processes. Research indicates that microbial adaptation to dietary changes can vary in time, with some adaptations occurring rapidly while others may require extended periods for a stable microbial response to be observed (Beauchemin et al., 2008;

McAllister and Newbold, 2008). Additionally, the time required for biochar adaptation is crucial to unlock its full potential for reducing methane emissions. The study by Inthapanya et al. (2012) demonstrated that the most significant reduction in methane production occurred in rumen fluid from animals that had been adapted to biochar. This suggests that biochar's effectiveness in mitigating methane may increase over time as the rumen microbiome adjusts to its presence.

6.4.2 Biochar and product X combined

The combination of product X and biochar within the high forage diet (trial 2) showed the lowest methane, carbon dioxide, and total gas production values at the end point of 24 hours incubation compared to other treatments, suggesting different mechanisms for methane reduction. Although this outcome was not statistically significant and the numerical ranking of treatments was somewhat different with cumulative methane outcome, it´s still might indicate synergistic actions of biochar and product X unique mechanisms that lead to a more potent methanogenic effect, activating methanogens differently. Similar symbiotic effects of biochar when applied with other methanogens were revealed in both *in vitro* (Leng et al., 2012a) and *in vivo* (Leng et al., 2012b) studies. For instance, the *in vitro* study showed that biochar (1%) combined with potassium nitrate and urea reduced methane production by 40-49%, while biochar applied alone had a less pronounced effect, reducing methane by 13% (Leng et al., 2012a). The *in vivo* study (Leng et al., 2012b) demonstrated a similar effect of symbiotic methanogenic activity of biochar (0.6%), showing lower methane production when combined with potassium nitrate (41%) versus biochar alone (22%).

Biochar increases the rumen's surface area for microbial habitats, potentially enhancing methane oxidation by favoring methanotrophs (Leng et al., 2012b). Nitrate acts as an alternative hydrogen sink, competing with methane production processes (Zhao et al., 2018). Their combination could lead to a more significant reduction in methane by merging biochar's structural benefits with nitrate's chemical influence on rumen fermentation. This dual approach maximizes methane mitigation by both altering the microbial community favourably and providing a more efficient hydrogen utilization pathway, thus amplifying the methanogenic effect of their combination. Although we are not allowed to disclose properties and composition of product X, based on Leng et al. 2012b outcomes, it can be suggested that biochar could serve as a potential accelerator for anti-methanogenic additives (with hydrogen sink effect).

6.4.3 Forage plant species

Grass silage decreased methane total production relative to silage mixtures. However, when methane production per digestible dry matter (DM) was considered, the difference was not evident. Though forage plant species had no major effects on total ruminal methane production in the current *in vitro* study, there were differences in methane formation kinetics. For cumulative methane production, results indicated a diet-time interaction throughout the 24-hour incubation period. The cumulative methane production was consistently lower for pure grass silage relative to silage mixtures throughout the whole 24h incubation period. This might be due to its higher fiber content that leads to more gradual fermentation (Van Soest 1994; McDonald et al., 2011). McDonald et al. (2011) explain that the rumen microbes responsible for fiber digestion, including cellulolytic bacteria, have a longer lag phase before beginning effective fermentation, which results in the gradual and consistent production of methane. Additionally, Van Soest (1994) suggests that the physical structure of plant cell walls in grasses provides a complex substrate that is not easily accessible to rumen microbes, further contributing to the slower fermentation rate.

The initial 8 hours showed higher cumulative methane production with red clover treatment that is in line with higher digestion and fermentation rate of red clover silage compared to grass silage-based diets observed *in vivo* (Kuoppala et al. 2009, Dewhurst, 2013). This effect is attributed to its readily fermentable non-fiber carbohydrates that provide an immediate substrate for microbial digestion (Broderick, 1995) and its isoflavone content which support faster degradation of fibrous material (Harlow et al., 2020).

From the 9th hour onward, significant disparities in cumulative gas production emerged between faba bean and maize treatments, with faba bean showing lower methane production, a trend that increased until the end of the incubation period. The lower methane production outcome of faba bean silage compared to maize silage, aligning with their D-value and digestibility rates; maize silage's higher digestibility due to abundant starch increases methane production rate (Janssen, 2010; Hristov et al., 2013). Furthermore, maize silage, due to its high starch content, undergoes rapid fermentation, potentially increasing methane production through enhanced propionate production, an alternative hydrogen sink (Hristov et al., 2013). In contrast, faba bean's nutrient profile, including its fiber content, might lead to a slower

fermentation rate, resulting in less methane production over time due to the gradual release of fermentable substrates (McAllister et al., 2011). Additionally, the high protein content associated with faba beans might shift fermentation towards ammonia production, since protein fermentation typically yields less methane than carbohydrate fermentation (Hristov et al., 2013), but it was not the case with red clover silage outcome in current study.

6.4.4 Forage-to-concentrate ratio

Although high forage diets are attributed to higher methane production than low forage diets (Aguerre et al., 2011; Bayat et al., 2017), the current study didn't reveal any statistically significant differences in methane production between high and low F:C ratios as hypothesized. The Aguerre et al. (2011) study demonstrated that adjusting F:C ratio in dairy cow diets from low 47:53 (NDF 313 g/kg DM; starch 290 g/kg DM) to high 68:32 (NDF 383 g/kg DM; starch 200 g/kg DM) increased methane emissions from 538 to 648 g/cow per day, indicating that higher forage content leads to increased methane production. However, current study showed no significant differences in methane production across diets with low (45% grass silage; NDF 407, CP, 189, starch 179 g/kg DM) and high (65% grass silage; NDF 453, CP, 179, starch 114 g/kg DM) F:C ratios. This contradiction might be due to relatively low starch content in 45:55 F:C diet. It has been proposed that a dietary starch content of 20-22% in diet DM is required to mitigate ruminal methanogenesis (Vanhatalo and Halmemies-Beauchet-Filleau, 2020) and our low forage diet remained below this threshold.

6.5 Limitations and need for further research

The observations from the current study indicate that a 24-hour incubation period may not suffice to reveal the full potential of biochar in mitigating greenhouse gas emissions. To fully assess biochar's potential to reduce methane emissions in ruminants, extending the incubation period to 48 hours or more with continuous culture systems is crucial. This longer duration is necessary to account for the adaptation of the rumen microbiome to biochar, allowing for a more accurate measurement of its long-term effects on methane mitigation. Short-term incubations may not fully capture the adaptation period necessary for the rumen microbiome to adjust to biochar, as seen in Inthapanya et al. (2012), where the most substantial reduction in methane was observed in rumen fluid from animals adapted to biochar in the period of 21 days. This finding underscores the necessity of longer-term studies to truly understand biochar's impact on methane production. *In vivo* verification is essential to observe biochar's real-world effectiveness, bridging the gap between controlled laboratory experiments and the complex dynamics of the rumen environment in live animals.

Additionally, there's a pronounced need for detailed studies exploring why biochar with an acidic pH exhibits a more significant effect on methane reduction compared to its alkaline counterparts. Preliminary research suggests that the pH of biochar influences its interaction with the rumen microbiota and fermentation processes, potentially affecting its methane mitigation capabilities. Understanding the mechanisms behind this pH-dependent effect could guide the optimization of biochar as a feed additive for methane reduction, tailoring its production to maximize environmental benefits.

Furthermore, greater variation in fiber and starch content within the diets would likely offer a more distinct understanding of biochar’s impact across different silage types and F:C ratios. Due to the simultaneous measurement of hydrogen and methane in GasEndeavour system, increases in hydrogen levels may mask reductions in methane production, affecting the accuracy of our results.

7 Conclusion

Spruce biochar demonstrated no significant effects on digestibility or methane production across different silage species (grass, red clover, maize, and faba bean) *in vitro*. There was no dose dependent response. While there were diet-time interactions observed in cumulative methane production, forage species did not influence methane production when adjusted per gram of digestible dry matter at the 24-hour time point. Additionally, neither biochar nor product X significantly impacted methane production across different F:C ratios. Furthermore, biochar did not significantly affect the final rumen pH or the total volatile fatty acid content, nor the molar proportions of acetic or propionic acids across silage types or F:C ratios (65:35 and 45:55). To enhance the understanding of biochar's role in rumen fermentation and methane emission across various diets, we propose extended incubation time along with continuous culture *in vitro* as well as validation *in vivo*. Evaluating the rumen microbiome's changes and possible resilience and stability in response to biochar, considering its unique physical and chemical properties, will also contribute significantly to understanding of how biochar interacts with different dietary compositions.

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Appendices

Appendix 1 Biochar EBC certified product list

Carbofex Oy EBC certified product list 2023

Product list

as of 30/03/2023

Carbofex Oy
Kaarnakatu 1
37150 Nokia
FI

ID-Number: **70783**

This product list is only valid in combination with a valid certificate downloaded from EASY-CERT. Products registered after this product list was issued are not listed below. They will be published subsequent to being certified at www.EASY-CERT.com/CH/70783

Products

Products	*Activities*	*Standards*	*Status*	*valid until*
Biochar Carbofex Natural - EBC AgroOrganic - Batch ID: ba-fi-37-1-3 - Production: 26.09.2022 - 25.09.2023	Storage Production Export	European Biochar Certificate	Recognized	31.12.2023
Biochar Carbofex Natural - EBC Feed - Batch ID: ba-fi-37-1-3 - Production: 26.09.2022 - 07.12.2022	Storage Production Export	European Biochar Certificate	Recognized	31.12.2023
Biochar Carbofex Natural - EBC FeedPlus - Batch ID: ba-fi-37-1-3 - Production: 08.12.2022 - 25.09.2023	Storage Production Export	European Biochar Certificate	Recognized	31.12.2023

EASY-CERT

bio.inspecta AG/q.inspecta GmbH
Ackerstrasse
CH-5070 Frick
Phone+41 (0)62 865 63 00
www.bio-inspecta.ch

q.inspecta