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Opportunities for Reduction of Methane Emissions by using different Feed Ingredients in Ruminant Nutrition

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Absztrakt

A globális felmelegedés veszélyének fokozódásával az állattenyésztési ágazat megvizsgálja, hogyan csökkenthetik a különböző takarmány-adalékanyagok az üvegházhatású gázok, nevezetesen a metángáz kibocsátását. A különböző irodalom áttekintései olyan új és innovatív tápanyag-kiegészítéseket tárnak fel a kérődző fajok napi adagjában, amelyek jelentősen csökkentik a metánkibocsátást az üvegházhatású gázok globális csökkentése érdekében. A metántermelést kiváltó enzimet elnyomó takarmány-adalékanyagok, valamint azok a takarmány-adalékanyagok, amelyek a bendő mikrobiomáját módosítják, mint például a különféle algák, takarmányok és specifikus illóolaj-keverékek, csak néhány a megoldások közül, amelyekről ebben az áttekintésben szó lesz. A forgalomban lévő engedélyezett takarmány-adalékanyagokat, amelyek könnyen hozzáférhetőek és hatásukat mutatják, szintén értékelni fogják. Különböző kísérleti módszereket alkalmazó in vitro és in vivo vizsgálatokról lesz szó.

Abstract

As the threat of global warming escalates, the livestock industry looks into exploring how different feed additives may reduce greenhouse gas emissions, namely methane gas. The reviews of various literature explore new and innovative nutritional additions in the daily rations of ruminant species that help reduce methane emissions significantly for the benefit of reducing greenhouse gases globally. Feed additives which suppress the enzyme that triggers methane production as well as those that modulate the rumen microbiome such as various species of algae, forages and specific essential oil blends are only some of the solutions that will be discussed in this review. Authorised feed additives on the market that are readily available and showing their effects will also be evaluated. In vitro and in vivo studies using different methods of experimenting will be discussed.

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1. Introduction

The phenomenal population growth in the world and ever-growing needs of its people place a huge demand on a whole wide range of animal products. Ruminant livestock such as cattle, buffalo, sheep and goats form the most popular and primary animals meeting such human demands. Ruminants play an important role biologically and economically as they turn forage and other feeds into high-quality and valuable protein sources of food.

Ruminants compared to other animals have a digestive system that allows for an optimised conversion of energy from plant material. Their digestive system, namely the rumen, through anaerobic and methanogenic processes is designed to ferment feedstuffs and generate precursors for energy that can be used by the animal. Bacteria, archaea, protozoa and fungi in the rumen microbiome carry out complex interactions that enhance the digestion of feedstuff in ruminants[1]. These microorganisms produce end products that are utilised either directly by the host or by other microorganisms as energy sources.

During the digestion of feed, carbon dioxide (CO2), methane (CH4), and hydrogen sulphide (H2S) are produced in the rumen[2]. Depending on the composition and the digestibility of the feed the rumen microbes produce quantities of CH4 which is then belched out. CH4 is a well-known potent greenhouse gas that traps heat and has a warming effect on the atmosphere[3]. The emission of CH4 from ruminant livestock must be minimised and controlled as CH4 is the key contributor to the formation of ground-level ozone, and therefore large quantities of CH4 is detrimental to the environment and life[4].

The ruminant diet consists mainly of cellulolytic plants such as straw, hay, silage and grass which are broken down by cellulolytic bacteria. Secondary metabolites in plants like tannins, saponins, terpenoids and flavonoids have been shown to alter the rumen microbiome, CH4 synthesis and proportions of volatile fatty acids in the rumen[5]. This may lead to alterations in the rumen microbiome of the composition and diversity of the methanogen community. Some rumen microbe species have been shown to improve the degradation of protein and fibre. A reduction of feed energy loss as methane gas can also be seen leading to a reduction in methane gas. Studies show that condensed tannins have a great anti-methanogenic effect as a result of affecting rumen bacteria and methanogens. Saponins tend to disrupt the rumen protozoa membrane which leads to decreasing the number of methanogenic archaea and protozoa. Essential oils impair the energy metabolism of archaea due to their volatile constituents of terpenoid and non-terpenoid origin. It has been shown that up to 26% in methane emission reductions have been seen in ruminants due to this characteristic. In vitro studies suggest evidence for the significant potential of flavonoids as a compound in reducing methane emissions.

Due to the various compositions of feed additives many not just plant supplements can modulate the rumen microbiome and shift its function towards an antimethogenic role. The enzymes that catalyse methane conversions are known as methyl-coenzyme M reductase and methane monooxygenase[6]. Certain feed additives such as Bovaer® comprising of 3-nitrooxypropanol can inhibit these enzymes significantly decreasing methane emissions[7].

Therefore, influencing the diet of ruminants may be considered the most effective and realistic way to decrease CH4 emissions and increase nitrogen utilisation efficiency practically. The manipulation of ruminant diet in decreasing CH4 emissions according to feed additives experimented in vivo and in vitro studies will be discussed further in detail.

2. Objectives

It is quintessential to provide better understanding, draw evidence based strong inferences, make comparisons and analyse trends as feasible in this study on CH4 emissions. The intent is to provide comprehensive understanding and inferences from the results of multiple studies and research conducted in recent years on the topic of CH4 emission in ruminants. The key objectives are to:

- Identify opportunities for reduction of CH4 emissions from using different feed ingredients in the nutrition of various ruminants of difference species and ages.
- Find which kind of feed additives play important roles in the mitigation of CH4.
- Summarise the effective ways for reduction of CH4 emissions with solutions.
- Presumably feed additives containing high amounts of plant secondary metabolites will have the greatest impact in reducing CH4 emission.

3. Literature Review

Several experimental research provide valuable knowledge on various forages, plant marcs, essential oils and extracts, which are reviewed in detail. Information on bacteria, fungi and insects is collected and evaluated as well. Additionally, research findings on authorised feed additives that are licensed and on the market are also reviewed in this section.

3.1 Forage

90% of the ruminant diet predominantly consists of roughage which includes leaves, grass, silage and crop residues. Forage being most of the feed for all ruminants plays a key role in their nutrition and overall animal health[8]. The following are opportunities in which changes can be made to the forage diet.

3.1.1 Sainfoin

In an in vitro study, sainfoin was co-ensiled with alfalfa in five rations. The results showed that depending on the proportion of sainfoin CH4 was reduced with dry matter digestibility being slightly negatively affected as well as suppressing silage proteolysis. Therefore, by adding sainfoin a high-quality legume silage is produced as well as mitigating rumen CH4 emissions[9].

3.1.2 Fresh and ensiled Paulownia hybrid leaves

In an in vitro study using ruminal fluid, Paulownia leaves fresh and in silage form were investigated for their CH4 mitigating effects[10]. It was shown that while ensiling the Paulownia leaves the phenolic acids and flavonoids content increased. Cellulolytic bacteria such as Fibrobacter succinogenes, Butyrivibrio fibrisolvens, and Prevotella spp. part of the gut microbiota[11] were shown to be higher in the fresh Paulownia leaves and silage group compared to the alfalfa silage group. The total archaea count was also lowest in Paulownia silage group, intermediate in the fresh Paulownia leaves group and highest in the alfalfa silage group. Therefore, it can be concluded that fresh Paulownia leaves and in silage form can decrease CH4 production by inhibiting methanogens and by improving the fermentation characteristics of the rumen.

A similar experiment was carried out with ensiled Paulownia leaves in vitro using the RUSITEC system and cannulated lactating dairy cattle[12]. As the dose of Paulownia leaves was increased a proportional decrease in CH4 concentration was observed. It was also observed that ruminal propionate, isovalerate and valerate concentrations were increased. There was also an increase in rumen protozoa and bacteria, but archaea were decreased. The overall CH4 production was decreased by 11% when compared to the control as seen in figure 1.

Item	Treatments[a]		SEM	*P*-value[b]
	CON	PLS		
Microbial populations				
*Ruminococcus flavefaciens**	1.18	10.77	2.79	0.09
*Fibrobacter succinogenes**	0.13	0.32	0.04	< 0.01
*Streptococcus bovis**	1.07	4.61	0.51	< 0.01
Prevotella spp.*	3.81	12.91	0.90	< 0.01
*Butyrivibrio proteoclasticus**	2.19	9.97	1.93	0.04
*Ruminococcus albus**	0.36	0.39	0.45	0.74
*Butyrivibrio fibrisolvens**	0.36	2.82	0.51	0.01
Lactobacillus spp.*	0.64	0.58	0.04	0.46
*Megasphaera elsdenii**	3.98	16.64	1.82	< 0.01
Total bacteria, × 10^9/mL	7.15	6.86	0.20	0.51
Total archaea, × 10^8/mL	6.28	5.28	0.19	< 0.01
Methanobacteriales, × 10^8/mL	4.33	3.44	0.15	< 0.01
Methanomicrobiales, × 10^7/mL	3.82	3.21	0.13	< 0.01
Dry matter intake	23.2	22.9	0.08	0.07
Total-tract digestibility [c], g/kg DM				
DM	631	618	4.42	0.15
OM	660	654	5.34	0.61
NDF	497	514	10.4	0.46
CP	616	584	6.07	< 0.01
EE	696	747	11.1	0.02
CH_4, g/d	459	410	9.80	< 0.01
CH_4, g/kg DMI	22.1	19.5	0.29	< 0.01
CO_2, g/d	11,403	12,008	203	0.97
CO_2, g/kg DMI	504	511	7.38	0.74

[a]*CON* control diet, *PLS* paulownia leaves silage diet; the percentage means of how many percentages of alfalfa was replaced with paulowina silage
[b]The results are considered to be significantly different at $P \leq 0.05$
[c]*DM* dry matter, *OM* organic matter, *CP* crude protein, *EE* ether extract, *NDF* neutral detergent fiber
*Abundance ($\log_{10}$ number of copies of *rrs* gene/mL of rumen sample)

Figure 1. Table of bacteria, methanogens, methane production and digestibility of Paulownia leaves study[12].

3.1.3 Morinda citrifolia leaves and fruits

Herbs are readily available and were also studied for their effects on mitigating methane emissions when added to ruminant diets. In this in vitro study the leaves and fruits of Morinda citrifolia, a type of herb, containing tannins and saponins were evaluated[13]. The herb in leaf (MCL) and fruit (MCF) form were added to incubation bottles containing Pennisetum purpureum grass. The results showed that compared to the control, the trial with Morinda citrifolia leaves has the highest gas production but with a decrease in methane gas production as seen in figure 2. This shows that the addition of Morinda citrifolia leaves added to a diet containing Pennisetum purpureum has the potential to decrease CH4 emission as well as improving the overall feeding value.

Treatment	Total gas (ml)	Methane (ml)	Methane (%gas)
R0	70.3[a]	23.3[a]	29.3[ab]
R1	68.3[a]	20.9[a]	30.6[b]
R2	125.0[d]	38.1[c]	30.5[ab]
R3	87.0[b]	22.9[a]	26.3[a]
R4	111.8[c]	33.9[b]	30.3[ab]
R5	111.3[c]	33.1[b]	29.7[ab]
R6	111.5[c]	33.5[b]	30.0[ab]
R7	109.8[c]	33.1[b]	30.2[ab]
R8	126.3[d]	36.5[bc]	28.9[ab]

R0: 100% PPG, R1: 90% PPG + 10% MCL, R2: 80% PPG + 20% MCL, R3: 70% PPG + 30% MCL, R4: 60% PPG + 40% MCL, R5: 90% PPG + 10% MCF, R6: 80% PPG + 20% MCF, R7: 70% PPG + 30% MCF, and R8: 60% PPG + 40% MCF.

Figure 2. Total gas production, CH4 production and concentration from dietary treatments with Morinda citrifolia leaves [13].

3.1.4 Olive leaves

In another in vitro study, two Hanwoo cows fed a basal diet of Timothy hay and corn were cannululated and the rumen fluid collected. The results showed that CH4 production decreased at the 12h mark of in vitro fermentation and the proportion of Fibrobacter succinogenes, Ruminococcus albus, and Ruminococcus flavefaciens also known as cellulose-degrading bacteria increased but decreased at 24h as in figure 3 [14].

Items	Fermentation Time (h)	Control	5% *Olive leaves*	SEM [1]	*p*-Value
Absolute abundance [2]					
Total bacteria	12	3.17 ^{a}	2.38 ^{b}	0.15	0.0212
	24	4.65 ^{a}	2.70 ^{b}	0.38	0.0216
Fungi	12	34.63	33.69	10.90	0.9543
	24	5.52 ^{a}	0.66 ^{b}	0.70	0.0079
Ciliate protozoa	12	1.45	3.82	0.85	0.1184
	24	0.97	1.09	0.43	0.8569
Methanogenic archaea	12	10.15	8.87	0.90	0.3712
	24	16.60 ^{a}	5.84 ^{b}	0.36	<0.0001
Relative proportion, % total bacteria					
Fibrobacter succinogenes	12	10.54	10.98	1.44	0.8313
	24	13.20 ^{a}	0.53 ^{b}	0.38	<0.0001
Ruminococcus albus	12	4.27 ^{b}	24.12 ^{a}	0.54	<0.0001
	24	5.29 ^{a}	2.23 ^{b}	0.34	<0.0001
Ruminococcus flavefaciens	12	0.86 ^{b}	1.02 ^{a}	0.03	0.0012
	24	0.66	0.63	0.02	0.2469
Prevotella ruminicola	12	26.22 ^{b}	31.04 ^{a}	1.48	0.0349
	24	38.97 ^{a}	28.09 ^{b}	1.07	<0.0001
Butyrivibrio fibrisolvens	12	1.99 ^{a}	1.17 ^{b}	0.08	0.0012
	24	2.78 ^{a}	1.26 ^{b}	0.21	0.2469
Butyrivibrio proteoclasticus	12	0.36 ^{a}	0.26 ^{b}	0.01	<0.0001
	24	0.46 ^{a}	0.32 ^{b}	0.01	<0.0001
Anearovibrio lipolytica	12	0.26 ^{b}	1.16 ^{a}	0.08	<0.0001
	24	0.93 ^{b}	4.34 ^{a}	0.27	<0.0001

[1] SEM, standard error of the mean. [2] Total bacteria, $\times 10^{10}$ copies/mL of ruminal fluid; fungi, $\times 10^{6}$ copies/mL of ruminal fluid; ciliate protozoa, $\times 10^{9}$ copies/mL of ruminal fluid; methanogenic archaea, $\times 10^{9}$ copies/mL of ruminal fluid; ^{a,b} means (n = 3) with different superscripts within a row differ significantly ($p < 0.05$).

Figure 3. In vitro rumen fermentation conditions using Olive leaves and its effect on rumen microorganism populations[14]

3.1.5 Cymbopogon citratus (CC), Matricaria chamomilla (MC) and Cosmos bipinnatus (CB)

An in vivo study using three herbs such as “Cymbopogon citratus (CC), Matricaria chamomilla (MC) and Cosmos bipinnatus (CB)” were provided to eight Charolais x Brown Swiss beef cattle kept in respiration chambers. It was concluded that CC and CB had significantly reduced CH4 production by 33% and 28% respectively while MC had no promising change. However, a further experiment took place where CC levels were increased exceeding the 2% dry matter intake, CH4 emissions were reduced but this was at the expense of decreasing digestibility.

3.1.6 Oats

The effect of oats was studied on sixteen lactating Nordic Red dairy cattle given a grass silage-based diet in an in vivo experiment. A respirator chamber was used in the model of GreenFeed system to measure the methane emissions. The results showed as oats gradually increased in the diet, CH4 emissions linearly decreased without having any production loss[15].

3.1.7 Hazel leaves

In an in vivo study, tannin-rich hazel leaves were supplemented to twenty lactating dairy cattle. The basal ration was mixed with concentrates given as well to observe for CH4 mitigating effects when supplemented with hazel leaves[16]. The experiment was done in open-circuit respiration chambers for 2 days. The results showed that lower feed intake and digestibility were observed when hazel leaves were given. This might have been due to the reduced feeding value of hazel leaves compared to when alfalfa was given. The results showed a huge potential to mitigate emissions of CH4 without any negative impact on the animal's performance at the same time.

3.1.8 Lespedeza cuneata hay

An in vitro followed by an in vivo study was carried out on four adult Dohne Merino sheep to study the effect of feeding Lespedeza cuneata hay containing high amounts of condensed tannins and its effect on mitigating CH4 emissions. For the in vitro study all four sheep were ruminally cannulated and for the in vivo study were placed in an open circuit respiration system for 6 days measuring the CH4 emissions continuously over 24h period. The effect of different amounts of hay was studied. As the inclusion level of this hay increased from 60% to 90% the CH4 emissions decreased. The results showed that when giving L. cuneata on a dry matter basis at 60% resulted in the highest reduction in methane emission by at least 21%. It can be concluded that to improve a diets dry matter digestibility L. cuneata could be added thereby increasing production from sheep as well as reducing methane emissions[17].

3.1.9 Gentiana straminea

An in vivo study was carried out similarly on thirty-two 5-week-old male Simmental calves given a basal diet consisting of alfalfa and oat hay, some concentrates, supplemented with different amounts of Gentiana straminea. The experiment was carried out in an open circuit respiration chamber for 5 days during which CH4 emissions were recorded. The results showed that Gentiana straminea could decrease CH4 emission without negative effects on the calves' health[18].

3.2 Plant marc (skins, seeds and stems)

3.2.1 Grape marc

Grape marc consists of the waste leftover of skins, seeds and stems of grapes composed together from the remains of wine production. In this in vitro ruminal study thirty-two Holstein dairy cattle early in their lactation period fed with perennial ryegrass basal diet were supplemented with red or white grape marc. The results showed that there was a decrease in CH4 emissions by 15% but this was at the expense of decreased milk production by 10% [19]. CH4 emissions were successfully mitigated due to the high concentrations of lignin and fat contents of grape marc however there was a significant decrease in milk production due to the reduced metabolisable energy taken in by the animal.

3.2.2 Grape pomace

Similarly grape pomace supplementation was studied by rumen fluids collected from four rumen-fistulated sheep before morning feeding. The study showed total ruminal gas produced was reduced as well as the methane production. There was evidence of nitrogen degradation, and the number of methanogenic archaea was significantly lowered. When Lactobacillus plantarum a “good bacterium” was added together with grape pomace there was a total gas reduction, so methane production was reduced and there was a significant increase in the digestibility of the silage. Therefore, grape pomace together with Lactobacillus plantarum when added to silage shows a great synergistic effect[20].

3.2.3 Indian gooseberry pomace

An in vitro and in vivo study took place on three fistulated Murrah buffalo bulls and ten lactating buffaloes respectively studying the effects of Emblica officinalis fruit pomace also known as Indian Gooseberry which is waste from fruit processing plants[21]. However, this compound is rich in polyphenolic compounds which can be seen as promising in reducing CH4 emissions. The results in vitro showed that CH4 production was decreased in higher doses. Sulphur hexafluoride (SF6) tracer technique was used in the in vivo trial so methane emissions from breath samples were recorded. The results from the in vivo study showed that not only was the CH4 production lower than the control group but there was improved milk yield and milk production efficiency. These results show great potential in using compounds with great use that would have been disposed of otherwise.

3.3 Essential oils/ extracts

3.3.1 Walnut (Juglans regia) leaf ethanolic extract

In an in vitro study, an increasing amount of walnut leaf ethanol extract (WLEE) was used on a corn or barley grain or both mixed diet[22]. Three cows given a basal diet containing alfalfa hay and concentrates were fistulated and ruminal fluid was collected to be analysed. The results showed that the addition of WLEE in increasing doses significantly decreased gas production and CH_4 emissions linearly. The microbial population of "Fibrobacter succinogenes, Ruminococcus flavefaciens and Ruminococcus albus" was reduced as a result of the addition of WLEE depending on the proportion of the basal diet[22].

3.3.2 Rhodophyta extract

In this study five Rhodophyta species extracts were supplemented in an in vitro study using rumen fluid donated by cannulated Holstein cows. These species include "Grateloupia lanceolata Kawaguchi, Hypnea japonica Tanaka, Pterocladia capillacea Bornet, Chondria crassicaulis Harvey, and Gelidium amansii Lamouroux" accordingly in the experiment. The results showed total gas production at 24h and 72h was increased due to the supplementation of the extracts. Further analysis took place using a Real-time Polymerase Chain Reaction (rtPCR) test which indicated that at the 24h mark, methanogens such as Ruminococcus albus and Ruminococcus flavefaciens decreased, while Fibrobacter succinogenes increased suggesting an overall decrease in methane gas emissions[23].

3.3.3 Acacia mearnsii

Acacia mearnsii bark containing large amounts of tannins were supplemented to cattle to study the long-term efficacy of the additive on methane emissions. To carry out the in vivo study respiration chamber was used. A diet consisting of a mixed ration supplemented with grass pellets, concentrates with a substitution of Acacia pellets in certain trials were given to twenty lactating Brown Swiss dairy cows. The results showed that A. mearnsii extract could act as a CH_4 mitigating supplement[24].

3.3.4 Linseed oil

To identify the effects of increasing linseed oil concentrations in ruminant diet[25], an in vitro study took place using twelve multiparous lactating Holstein cows with ruminal cannula. The results showed that the increase in linseed oil can linearly reduce enteric CH_4 emissions. At 2-

3% linseed oil the effects of CH_4 emissions can be mitigated up to 20% without impairing productivity[25].

3.3.5 Cottonseed meal

An in vivo study was carried out on British cross steers using the GreenFeed system to measure methane emissions when fed ad libitum low-quality bluestem hay with the supplementation of cottonseed meal. The results showed that feed intake increased with the protein supplementation and CH_4 emissions decreased according to the proportion of gross energy intake. This shows that supplementing low-quality forages with proteins helps decrease greenhouse gas emissions per gross energy intake[26].

A similar study took place using eight crossbred steers in respiration calorimetry when fed low, medium and high-quality forages supplemented with cottonseed meal. The results showed that the protein supplementation had no effect on methane gas production in the low-quality forage trial but increased dry matter, fibre, energy and protein digestion. It was seen that the methane production per unit of energy decreased as protein was supplemented to low, medium and high-quality hays showing that as the quality of forages increases, methane production could potentially decrease[27].

3.3.5 Palm oil

An in vitro and in vivo study took place on four rumen-cannulated heifers fed low-quality grass for rumen sampling and 3 days in respiration chambers to measure the methane gas production when supplementing palm oil. As the supplementation of palm oil gradually increased so did the ruminal concentration of propionic acid. However, the acetic and propionic ratio and butyric acid and isobutyric acid decreased accordingly. The daily methane gas production total was lower in diets containing palm oil than in the control without any supplementation. It is possible as stated by the study that for every 10g/kg PO in the diet, methane gas can be reduced by 4% without affecting the dry matter intake and apparent digestibility[28].

3.4 Seaweeds

3.4.1 Ecklonia stolonifera extract (brown algae species)

An in vitro study of Ecklonia stolonifera (E. stolonifera) extract was carried out to investigate the effect of the supplementation on methane gas productions and ruminal fermentation due to its phlorotannins that might be able to decrease methane gas production. In this experiment, one Holstein cow was cannulated and was fed timothy hay and concentrate. Timothy hay was used as the substrate and rumen fluid from the cow was both used for the in vitro fermentation which was also supplemented with E. stonifera extract at various concentrations. The results showed that total gas production increased, with methane emissions increasing. This contrasts with other in vitro studies done on brown algae extracts. A rtPCR test was carried out that showed that Fibrobacteri succinogenes populations decreased while Ruminococcus flavefaciens populations increased. Therefore, it can be concluded that E. stolonifera does not negatively affect ruminal fermentation with no impact on mitigating methane emissions[29].

3.4.2 Brown Algae

An in vitro study was carried out to investigate the effects of "brown algae phlorotannin derivatives such as phlorofucofuroeckol-A (PFFA), dieckol (DE), and 8,8'-bieckol (BE)", regarding methane gas production. Two Holstein cows were cannulated to be used as rumen fluid donors which were then incubated in vitro culture systems for 24h and 48h supplementation with each phlorotannin derivative. The results showed that the brown algae phlorotannin derivatives of PFFA, DE and BE were able to reduce methane emission in the rumen. Additionally, in the results in phlorotannin derivatives there was a positive correlation between the number of hydroxyl groups and ether linkages to the mitigation of methane emissions[30].

3.4.3 Asparagopsis taxiformis

A study was carried out to investigate the supplementation of Asparagopsis taxiformis and its effects on methane emissions. Two in vitro and two in vivo experiments were carried out on lactating dairy cows. The greenfeed system was used for in vivo experiments and rumen samples were collected to analyse the ruminal fermentation characteristics. In vitro results showed a decrease in methane emissions by 98% when A. taxiformis was added. In vivo studies also showed a significant decrease in methane emissions. It can be stated that overall, when A. taxiformis was added to the diet at 0.5% it can mitigate methane emissions greatly[31].

3.4.4 Asparagopsis armata

An in vivo study using the GreenFeed system evaluated the enteric CH4 emission production over 21 days of twelve post-peak lactating Holstein cows supplied Asparagopsis armata on an organic matter basis[32]. As seen in figure 4 due to the inclusion of seaweed into the diet there was a methane reduction of up to 67%. CH4 production by dairy cattle is reduced by up to 60%

when adjusted for milk production. As CH_4 emissions decreased hydrogen and carbon dioxide increased.

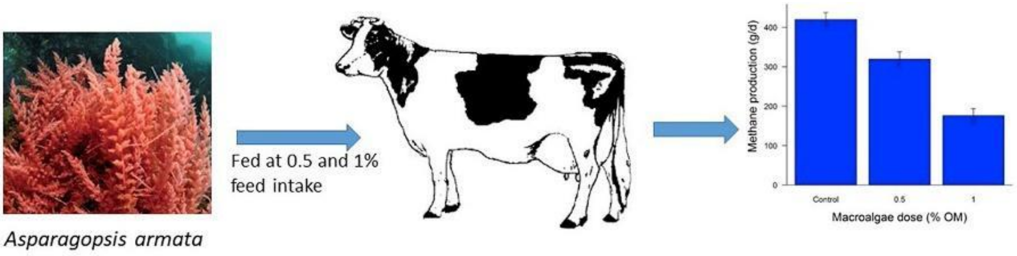

Figure 4. Negative correlation between CH_4 production (g/d) and macroalgae doses (% OM) [32].

3.5 Bacteria

3.5.1 Lactobacillus plantarum, Lactobacillus buchneri, Pediococcus pentosaceus

An in vitro ruminal fermentation study carried out using corn silage treated with bacterial inoculants comprising of "Lactobacillus buchneri (LB), Lactobacillus plantarum (LP), Pediococcus pentosaceus (PP)" with the concentration of 30%, 60% and 10% respectively have shown a reduction in CH_4 emissions[33]. These bacteria produce feruloyl acid esterases that can hydrolyse esterified phenolic acids from plant cell walls improving silage quality by decreasing the pH and increasing lactate production. This inoculum in the corn silage thereby facilitated microbial digestion of lignified fibre and made rumen fermentation produce more propionate as well as decrease its methane gas production.

The study previously mentioned consisting of grape pomace as a supplement alone or together with Lactobacillus plantarum A1 (Lp A1) can be discussed further in detail here regarding Lp A1. Lp A1 alone when added to alfalfa silage showed an increase in total gas and methane production when compared to silages without Lp A1. However, when grape pomace is added to the silage together with LP A1 it reduces the total gas and methane production of silage. This may be due to the tannins in grape pomace decreasing methane emissions as mentioned earlier[34].

3.5.2 Pseudomonas aeruginosa

In a different kind of study, "nitrate-dependent anaerobic methane oxidation (AMO)" in rumen fluid culture was made. This was done to investigate the organisms present in the rumen of dairy goats that carry out the process of denitrifying anaerobic methane oxidising (DAMO) in two enrichment culture systems. These two culture systems were supplied with only methane gas and $NaNO_2$. From the enrichment system Pseudomonas aeruginosa was isolated and was able to carry out the DAMO process on its own. Further in vitro rumen fermentation test was carried out with the isolated Pseudomonas aeruginoas and results showed that it was able to reduce CH4 emissions[35].

3.6 Fungi

3.6.1 Five white-rot fungi

In vitro fermentation of five white-rot fungi on corn straw were studied. These five species include "Pleurotus ostreatus, Lentinus edodes, Hericium erinaceus, Pleurotus eryngii and Flammulina filiformis"[36]. All fungi species studied, had a reduction in dry matter digestibility and total volatile fatty acid concentration and total gas produced as fermentation was prolonged. Except for F. filiformis, an in rumen fermentability was observed in all other fungal species trials. A reduction in methane emission was seen in the F. filiformis trial while no reduction in total gas production was observed in the L.edodes trial.

3.7 Insects

3.7.1 Acheta domesticus, Brachytrupes portentosus, Gryllus bimaculatus and Bombyx mori

Edible insects have a high nutritive value over the conventional ruminant feed. This study partially substitutes soybean meal for four different kinds of edible insects with high protein and fat contents[37]. The four insects include Acheta domesticus (A. domesticus) also known as adult house crickets, Brachytrupes portentosus (B. portentosus) also known as adult giant crickets, Gryllus bimaculatus (G. bimaculatus) also known as adult field crickets, and finally Bombyx mori (B. mori) also known as silkworm pupae. Through in vitro incubation, 25% of soybean meal was replaced by all four edible insects. The study showed that the inclusion of all four edible insects did not affect the following parameters: nutrient digestibility, rumen fermentation and volatile fatty acid production. The production of ammonia and nitrogen however did increase. Significant methane mitigation was seen in the addition of Gryllus bimaculatus and Bombyx mori by 18% and 16% respectively. Results show great possibility towards a more sustainable livestock industry due to the significant mitigation of CH4 emissions and by substitution of a lower-in-cost protein source yet with a high nutritive value.

3.7.2 Piper beetle powder

An in vitro study with rumen inoculum from four multiparous Saanen goats was studied for the effects of decreasing CH4 emission when Piper beetle powder (PBP) and/or sunflower oil

(SFO) was supplemented with the basal diet. The results showed that PBP alone or with SFO decreased CH4 production and the number of rumen protozoa significantly without compromising rumen fermentation parameters[38].

3.7.3 Silkworm pupae oil

An in vitro study on the effect of mitigating CH4 emissions using silkworm pupae oil was carried out on eighteen Mandya adult sheep fed a basal diet supplemented with 2% oil either daily or intermittently. The results showed a decrease in enteric CH4 emissions by 15-20% with no significant difference between the daily or intermittently fed groups [39].

3.7.4 Chitosan

A basal diet was supplemented with chitosan of different molecular weights in six different units of an in vitro study. The gas chromatography technique was used to analyse and measure the concentrations of methane emissions collected during the study. Illumina MiSeq platform was used to analyse the relative abundances of the bacterial community and to sequence the bacterial 16S rRNA genes. The results showed that propionate proportion was significantly increased while CH4 and acetate production were significantly decreased. The analysis showed a positive correlation between Prevotella to propionate production. Therefore it can be concluded that by promoting the growth of amylolytic Bacteroidetes and Proteobacteria instead of Firmicutes and Fibrobacteres which are fibrolytic, chitosan can reduce CH4 production[40].

3.8 Authorised feed additives

3.8.1 Mootral

Mootral a British-Swiss company developed a natural feed supplement called “Enterix™” or also known as “Mootral” on the market, consists of “garlic powder (Allium sativum) and bitter orange extracts (Citrus aurantium)” that works to reduce CH4 emissions from ruminants[41].

In this first study, eight Nordic Red dairy cows CH4 emissions were evaluated using open-circuit respiratory chambers on 4 consecutive days[42]. The results showed that compared to the control CH4 production decreased when diet included Mootral.

Another study on mootral took place to investigate its long-term effects by using the in vitro rumen simulation technique (RUSITEC) system. To study this an experiment on Mootral was conducted that lasted 38 days of investigation.[43]. The results showed that CH4 gas production was reduced possibly due to the isoflavanoids that mootral contains such as naringin. However, the production rate of CH4 returned to the initial amount from day 18 onwards. Mootral was able to affect the ruminal fermentation by increasing relative abundance of Methanomassiliicoccales and reducing the Methanomicrobia. However, this was a transient effect and therefore the methane reduction is only transiently available with the supplementation of mootral due to the abundances of bacterial families only slightly being affected. During the application of mootral, the increase in the concentration of pyridoxine (vitamin B6) was significant which highly benefits the animal.

3.8.2 Bovaer (3-NOP)

Bovaer is a feed supplement created by DSM with the aim to achieve a significant and immediate reduction of CH4 emission and claims to have done so in dairy cattle by 30% and in beef cattle by 45%[44]. It is also known as 3-Nitrooxypropanol (3-NOP). It comprises two ingredients nitrate and biobased alcohol that suppresses the enzyme that generates CH4. To the total mixed ration, when the lowest dose at 60 mg/kg DM of the total daily ration was added, it can reduce CH4 emissions produced by dairy cows by 22-35%. Furthermore, in most trials carried out, there was a 4% increase in the significance of the feed efficiency due to the increase in milk fat and protein. It could be seen that the effect lasted more than 100 days and was consistent with no sign of adaptation seen[45].

An in vitro study with eight ruminal cannulated cows took place that were given a plant protein diet with 3-NOP supplementation. During the 3-NOP trial, the rumen ammonium peak after feeding was lower than the control assuming it was due to the lower intake of digestible organic matter resulting in limited nitrogen and energy therefore causing a reduction in the synthesis of microbial amino acids[46]. Results showed the 3-NOP supplement to be highly effective in mitigating CH4 emissions with this lower-quality diet.

An in vivo study took place where 100 crossbred steers were allowed access to the GreenFeed emission monitoring system for 7 days while being supplemented with different doses of 3-NOP. The decrease in methane emissions was as follows when supplementing 3-NOP in low, medium, and high doses by 52%, 76%, and 63%, respectively.

Hydrogen emissions were also recorded showing a 5x increase while the ratio of acetate to propionate rumen fluid was analysed showing a decrease as 3-NOP was supplemented. It can be concluded that the other hydrogen-utilising pathways become more apparent as CH4 is inhibited in the rumen[47].

Another in vivo study took place where 3-NOP was supplemented to test if its efficacy was persistent when fed to early lactation dairy cows. A basal diet consisting of "35% grass silage, 25% corn silage and 40% concentrate" was given to sixteen multiparous Holstein Friesan cows during this study. For 5 days the dairy cattle were kept in respiration chambers where their total gas production was measured. In this study their lactation production and feed intake was also measured. The results showed that the responses such as feed efficiency, milk yield and dry matter intake were not affected at all. Methane production was decreased but at different levels according to the feed intake level most likely due to the different stages of lactation the animal was in. The results also proved that with the use of 3-NOP, organic matter, gross energy and total-tract digestibility of dry matter was increased compared to the control. Therefore, feeding 3-NOP to lactating dairy cattle does not have any negative impact but rather is an effective supplementation in reducing methane emissions [48].

Similarly, another in vivo study using the GreenFeed system took place where the inclusion rate of 3-NOP was studied on 49 multiparous Holstein cows. The results showed that compared to the control the enteric methane emissions decreased from 22% to 40%. The three highest doses 100, 150, and 200 mg/kg showed the maximum mitigation effect with no statistical difference among all three. Hydrogen gas production was also recorded with a 6 to 10-fold increase compared to the control trial. Carbon dioxide could be noticed as linearly increasing as the 3-NOP doses were increased. The production parameters of the cows such as dry matter intake and milk yield showed no significant change. The increase in concentration of de novo synthesis of short-chain fatty acids (SCFA), resulted in the increase of yield and milk fat concentration. Therefore, a suggested dose could be 100 mg/kg using the least amount of 3-NOP with the maximum mitigation effect[49].

4. Materials and Methods

4.1 Characterisation of Feed Ingredients

Feed ingredients are the component parts, or the constituents or the combination mixture that comprises the animal food. The composition of the feed, its type and quality play a vital role in the amount of CH4 emitted by the ruminant. Various feed ingredients in different compositions have been used to study their effect on CH4 emissions and their productivity.

The feed ingredients are the materials used in the design of experiments or clinical trials in understanding their effect on the emissions. In such studies, the amount of CH4 emission is measured as the primary metric and the measures of productivity are the secondary metrics, which together are the dependent variables or the outputs (Y). The materials of choice for a study are the independent variables or the inputs (X), of which the compositions are changed with reference to the control settings. It is important to recognize the presence of noise variables (N) during experiments of those whose effect can only be quantified or controlled to a certain extent. Certain independent variables remain constant (C) through the experimental study, thus having no or minimum effect on the dependent variables. Figure 5 shows an example of a schematic mapping of inputs and outputs for an experimental set-up. It is important to characterise the independent variables involved in a study. Table 1 summarises the feed additives used in the 'in vitro' studies and table 2 shows those used in the 'in vivo' studies.

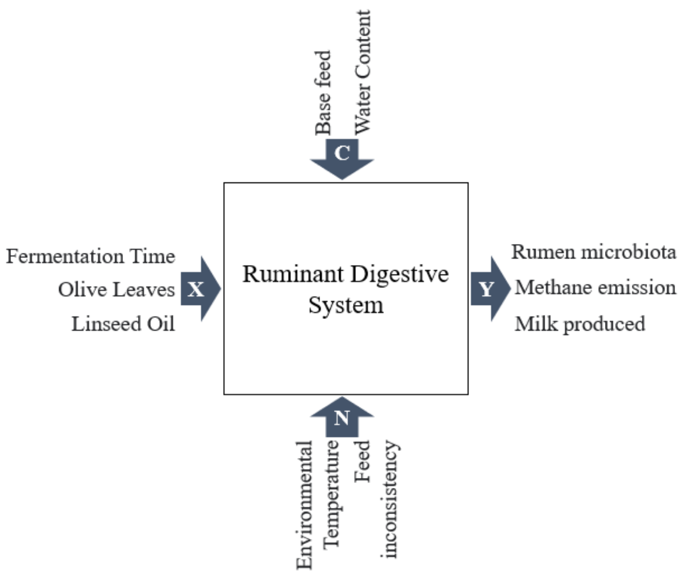

Figure 5. A typical mapping of the inputs and outputs in an experimental study for methane emission reduction

Table 1. Feed Additives used in the 'in vitro' studies.

Forage	Plant marc	Essential oils, Extracts	Seaweed
Sainfoin Olive leaves	Grape marc	Walnut leaf ethanolic extract	Brown algae species
Fresh and ensiled Paulownia hybrid leaves.	Grape pomace	Rhodophyte extract	Asparagopsis taxiformis
Mornida citrifolia leaves and fruits	Indian Gooseberry pomace	Acacia mearnsii	
Lespedeza cuneata hay		Linseed oil	
Bacteria	**Fungi**	**Insects**	**Authorized Feed Additives**
Lactobacillus plantarum	Five white-rot fungi (Pleurotus ostreatus, Lentinus edodes, Hericium erinaceus, Pleurotus eryngii and Flammulina filiformis)	Adult house crickets (A. domesticus), adult giant crickets (B. portentosus), adult field crickets (G. bimaculatus), and silkworm pupae (B. mori)	Mootral: garlic powder (Allium sativum) and bitter orange extracts (Citrus aurantium)
Lactobacillus buchneri			
Pediococcus pentosaceus		Piper beetle powder	3-nitrooxypropanol (3-NOP, Bovaer)
Lactobacillus plantarum A1		Silkworm pupae oil	
Pseudomonas aeruginosa		Chitosan	

Table 2. Feed Additives used in the 'in vivo' studies.

Forage	Plant marc	Essential oils, Extracts	Seaweed	Authorized feed additives
Cymbopogon citratus (CC), Matricaria chamomilla (MC) and Cosmos bipinnatus (CB)	Indian gooseberry pomace	Acacia mearnsii	Asparagopsis taxiformis	Mootral: garlic powder (Allium sativum) and bitter orange extracts (Citrus aurantium)
Hazel leaves		Cottonseed meal	Asparagopsis armata	
Oats		Palm oil		3-nitrooxypropanol (3-NOP, Bovaer)
Lespedeza cuneata hay				
Gentiana straminea				

The various plant feed ingredients are made of secondary plant metabolites such as terpenoids, phenolic compounds, alkaloids and sulphur-containing compounds as seen in figure 6. These phytochemicals have an inhibitory effect on methanogens, protozoa and other hydrogen-producing organisms which leads to significant CH4 mitigating effects[50].

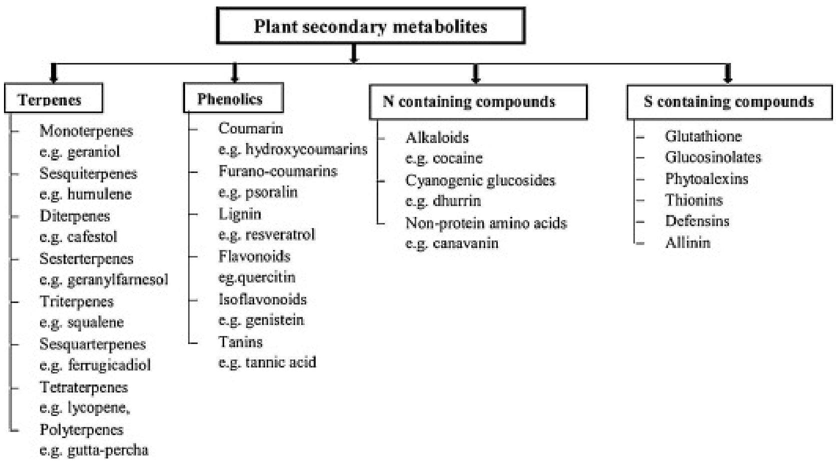

Figure 6. Categorisation of various plant secondary metabolites [51]

The bacterial ingredients and shift in rumen fermentation affects the rumen microbiota resulting in increase in propionate and reduction in CH4. The study also shows that edible insects were analysed to be rich in fat and protein. They also have an essential amino acid profile similar to soybean meal. Therefore, when substituting soybean meal for the tested insects the fermentation profile and nutrient digestibility remains unchanged. There was a reduction in methane gas production in G. bimaculatus and B. mori particularly by 18% and

16% respectively. Chitosan on the other hand was able to shift the microbiota towards more amylolytic bacteria such as Bacteroidetes and Proteobacteria that help reduce methane emissions.

Authorised feed additives such as mootral can be seen as a natural feed supplement similarly having secondary plant metabolites that have the characteristic of naturally mitigating CH4 production since it is made up of Allium sativum and Citrus aurantium. Bovaer on the other hand is made up of the synthetic ingredient called 3-nitrooxypropanol. The main mechanism of action is to suppress the enzyme known as methyl-coenzyme M reductase (MCR) which inhibits the formation of methane gas without any adverse impact on the rumen microbiota[52].

4.2 Method of In Vitro Study

The vitro method of study uses the "*in vitro gas production technique*" (IVGPT) set-up, which simulates the cows' ruminal fermentation of feedstuff. Therefore, when studying the production of methane emissions, we can use this method to observe the amount of gas produced under lab conditions[53]. Under these strict laboratory conditions, the main principle is to ferment feedstuff by adding naturally occurring rumen microbes. According to the needs of the study the feed materials are subjected to various treatments including an incubation at 39°C which runs typically from 24, 48, 72, 96 or 144 h. Figure 7 gives a graphical extract of an in vitro study.

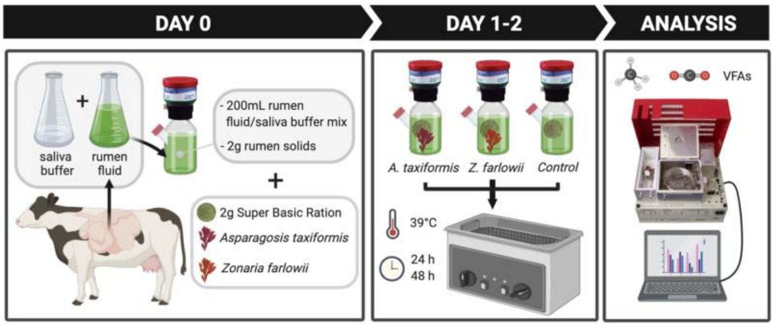

Figure 7. An example of in vitro study carried out using two algae species to measure CH4 gas production [54]

As we have seen in numerous studies mentioned previously rumen fluid is collected and this method is carried out by supplementing different feed additives. The total gas produced is then measured and analysed. In this case, we can then see how much methane gas was produced. The results from IVGPT experiments are known as the amount of CH4 per gram of dry matter. Different IVGPT systems are available for methane determination such as syringes, Rumen simulation technique (RUSITEC)and closed vessel batch fermentation. The latter two are what have been used most often in the studies previously mentioned. All in vitro methods require fresh rumen fluid obtained from ruminants that were fistulated.

4.3 Method of In Vivo Study

There are multiple different ways to carry out an in vivo study. The gold standard is the respiration chambers. The chamber collects all exhaled air produced by the animal and measures the amount of CH4 produced as seen in the studies mentioned earlier[53]. There are two main types of respiration chamber systems. There are the closed circuit and open circuit respiration chamber systems. Figures 8 and 9 show an open circuit system is used more often. Air is either pumped into the chamber from outside or through an air conditioning system. The flow and concentration of the air at the inlet and outlet of the chamber is calculated and analysed to give the CH4 production.

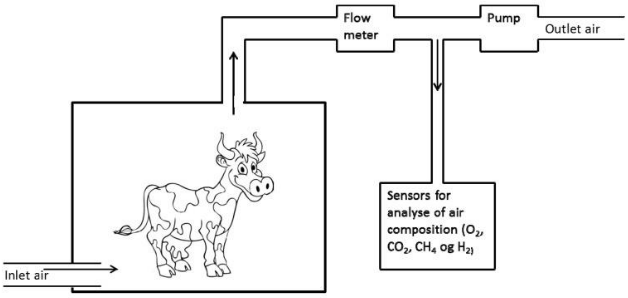

Figure 8. Illustration of an open-circuit respiration chamber [53]

Figure 9. Example for Respiration chambers used in vivo studies [53]

Another method most commonly used in the in vivo studies mentioned was the GreenFeed Pasture System as seen in figure 10. This is a turn-key system designed by C-Lock to measure gas fluctuations of CH4, CO2, O2 and H2 from individual animals[55]. It is also possible to determine herd averages when combining data from individual animals. Food acts as bait to attract the animals to visit the system multiple times per day. The data collected is automatically processed, giving an immediate report on the amount of total gas produced. The system is greatly advantageous as it allows for utilisation on the field. It allows the animals to roam freely as well as express their natural behaviour and habits.

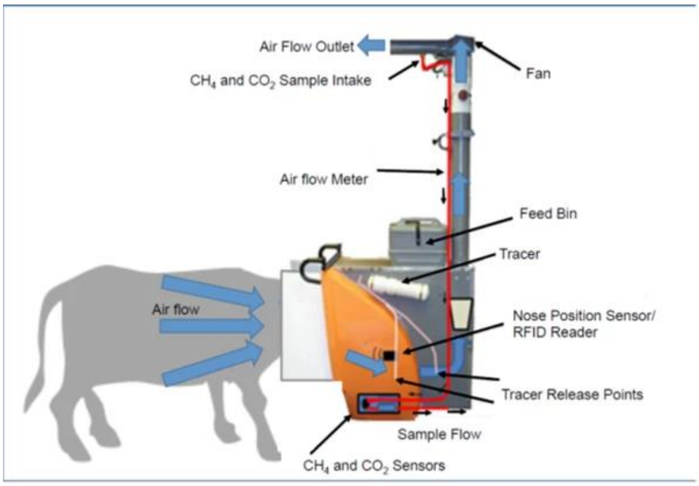

Figure 10. illustration of GreenFeed Pasture System [56]

Finally, the SF6 tracer technique is a method also used to collect and analyse methane emissions from ruminants. Its use is based on allowing the animals to free roam as seen below in figures 11 and 12. A tube is filled with SF6 gas which is then placed in animal rumen. Once secured the collection of gases can begin. The results are then analysed using gas chromatography.

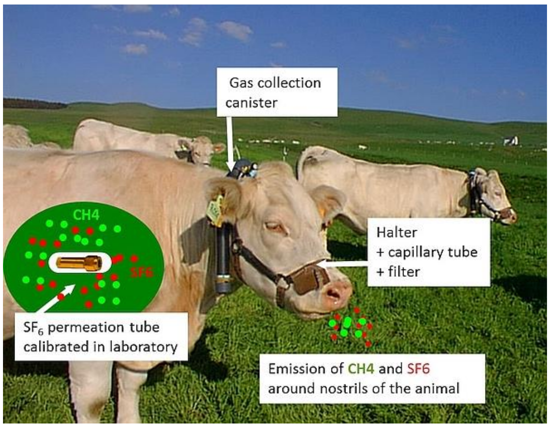

Figure 11. An illustration of the SF6 Tracer method [57]

There are advantages and disadvantages to all the methods mentioned in the in vitro and in vivo studies. In vitro methods allow for a strictly controlled environment to test out all conditions and feed additive possibilities. However, in real life, the animal is not a sterile and controlled being so the result may vary when done in the animal itself. There is a lack of information available on the ruminal microbiota stability in the RUSITEC system[10]. IVGPT in general only stimulates the fermentation of feed and does not allow for long-term adaptation of the ruminal microorganisms which is usually seen in ruminants. Therefore, it is suggested in vivo experiments should be carried out with an adaptation period of at least two weeks. Therefore, IVGPT technique should be only used to test out potential feedstuffs and conditions before a more expensive in vivo experiment is carried out.

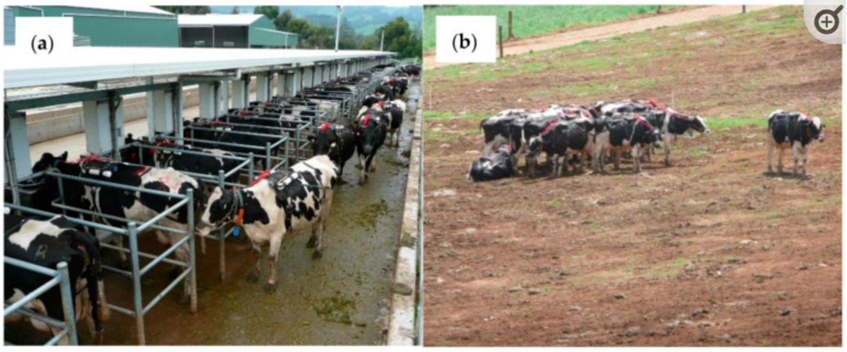

Figure 12. A study carried out using SF6 Tracer method [58]

Respiration chambers allow for a more accurate data collection of the amount of methane emissions produced by each animal due to the controlled environment and the stability of the instruments used. However, animals are not able to be kept in those chambers for extended periods due to welfare reasons. Therefore, it does not allow for the animals to exhibit its natural behaviours so results may vary according to real situations on the field. The GreenFeed system is a respiration chamber system that allows for a more flexible data collection on the field. Animals are allowed to come and go freely from the machine and a more accurate data collection is done. The SF6 tracer technique allows for the system to be on the pasture and allows for the animal to move freely. However large variations in results have been reported when the technique has been used. This could be due to background gases being identified and the type of flow restrictor being used. Windy or rainy weather conditions may also disturb the results being recorded. Therefore, in comparison to SF6 tracer techniques, respiration chambers are more reliable.

Overall, it is understood that based on the IVGPT analysis results, proceeding to the SF6 technique and respiration chamber method allows for a thorough animal experiment with reliable data.

5. Results

In the topic of forages many different feed additives were seen. In the in vitro studies, sainfoin was able to decrease methane emissions as well as suppress silage proteolysis while fresh Paulownia leaves and silage was able to decrease methane emissions by inhibiting various methanogens and improving fermentation characteristics of rumen. Morinda citrifolia leaves in vitro also showed mitigating methane emission effects. Olive leaves in vitro also showed a decrease in methane production at 12h while the proportion of cellulose degrading bacteria increased, however this decreased at 24h.

In the in vivo studies Cymbopogon citratus and Cosmos bipinnatus herb showed a significant decrease in methane production by 33% and 28% respectively. When oats were given as a feed supplement as its dosage increased the methane emissions reduced gradually without showing any production loss in the dairy cattle. Hazel leaves were also observed to decrease methane emissions but with lower feed intake and digestibility seen as well. Lespedeza cuneata hay had both in vivo and in vitro studies done that showed an increase in dry matter digestibility resulting in better production results and decreased methane production. Gentiana stramine supplemented in calves in an in vivo study showed a decrease in CH_4 production.

Grape marc is made of the components of the fruit itself such as the skins, seeds and stems. In this by-product, there are high concentrations of lignin and fat which causes CH_4 emissions to decrease resulting in a decrease in intake of metabolizable energy therefore leading to a decrease in milk production as well seen in this in vitro study. In the vitro study using grape pomace instead, showed when Lactobacillus plantarum was added together there was a synergistic effect of reduction in CH_4 and there was an increase in silage digestibility. While supplementing Indian Gooseberry pomade in vitro and in vivo the results were significantly great as it reduced methane emissions, improved milk yield and milk production efficiency.

In the in vitro study with walnut leaf ethanol extract, as its dosage was increased, methane production decreased, and microbial population shifted towards anti methanogenic bacteria. In vitro study using five Rhodophyta species showed a decrease in overall methane gas production. In vitro study of linseed oil showed that as concentrations increased, CH_4 linearly decreased. At 2-3% linseed oil, CH_4 was able to be reduced up to 20% without any adverse production results.

In vivo study using Acacia mearnsii bark showed that due to its increasingly high amounts of tannins, it was able to reduce methane emission significantly in the twenty lactating cows. An in vivo study using cottonseed meal showed that feed intake was increased with the protein supplementation and CH4 decreased according to the gross energy intake. This result shows significant evidence to believe that supplementing protein to low-quality forages helps in reducing CH4 emission. An in vitro and in vivo study took place supplementing palm oil that showed that for every 10g/kg PO in the diet, methane gas can be reduced by 4% without affecting the dry matter intake and apparent digestibility.

Multiple in vitro studies with brown algae were carried out however the first one using Ecklonia stolonifera extract showed an increase in methane emissions due to a shift in microbial population towards methanogenic bacteria. The next in vitro study that took place used three different phlorotannin derivatives of brown algae. The number of hydroxyl groups and ether linkages show positive correlation along with reduction in methane emissions in all three groups. In vitro and in vivo studies took place using Asparagopsis taxiformis, a red macroalgae. The results showed that even when added to the diet at 0.5% it was able to significantly reduce methane emissions. An in vivo study took place on Asparagopsis armata where the results showed a methane reduction of up to 67% and when adjusted for milk production CH4 reduced up to 60%.

The in vitro study used mixed inoculants of lactobacillus and Pediococcus bacteria to enable ester-linked phenolic acids released from plant cell walls, improving silage quality which shifted rumen fermentation causing a decrease in methane emission. These in vitro rumen fermentation results showed reduction in CH4 when supplemented with Pseudomonas aeruginosa. All five fungi species in the in vitro trials significantly decreased lignin and cellulose. However only F. filiformis species was able to reduce methane emissions.

Chemical analysis showed that the edible insects (Acheta domesticus, Brachytrupes portentosus, Gryllus bimaculatus, and Bombyx mori) were rich in fat by about 14–26%. The unsaturated fatty acids proportion of this was around 60-70%. These edible insects were also rich in protein, around 48–61%. The essential amino acids and amino acid composition of these edible insects were astoundingly similar to that of soybean meal. The in vivo study showed that including insects as a feed supplement did not have any adverse effect on production of volatile fatty acids and nutrient digestibility. Replacing 25% of the soybean meal used in the diet with edible insects had the advantage of potential reduction in CH4 production with no other negative effect. Significant methane mitigation was seen in the addition of Gryllus bimaculatus and Bombyx mori by 18% and 16% respectively.

In vitro study using piper beetle powder in goats showed a decrease in CH_4 production and rumen protozoa without affecting fermentation parameters. Another in vitro study used silkworm pupae oil in adult sheep showing when supplemented with 2% oil daily or intermittently, a decrease in enteric CH_4 by 15-50% was observed. An in vitro study carried out using chitosan as supplementation which showed that as propionate increased, CH_4 and acetate decreased. There was a positive correlation between propionate and Prevotella bacteria showing that chitosan can promote the growth of anti-methanogenic bacteria decreasing the overall CH_4 production.

In vivo study carried out by supplementing Mootral showed that CH_4 was able to be reduced. However, in an in vitro study only a transient reduction in methane emission was observed for 18 days. It did so by being able to shift bacterial rumen fermentation, increasing vitamin B6 concentration which is of high benefits to the animal.

3-NOP in vitro study showed that it was highly effective in mitigating CH_4 emission due to being able to limit nitrogen and energy causing reduced synthesis of microbial amino acids. An in vivo study showed that there was a decrease in methane emissions in low, medium and high doses by 52%, 76%, and 63%, respectively. Hydrogen emissions could also be observed as being 5x higher suggesting that other hydrogen utilising pathways became more apparent as CH_4 is inhibited in the trial. Another in vivo study took place that showed there was no effect on lactational parameters when supplementing 3-NOP and was still able to reduce methane emissions. The final in vivo study of 3-NOP showed there was methane emission decrease from 22% to 40% when compared to the control. All three of the highest doses of 3-NOP (100, 150, and 200 mg/kg) showed the maximum mitigation effect. Therefore, it would be wise to supplement with 100 mg/kg for the best feed efficiency.

6. Discussion

Many feed additives were discussed from plants containing high amounts of phytochemicals to bacteria, fungi and insects. All had different roles in ruminal fermentation processes that allowed for the overall mitigation in methane emissions. The most commonly seen feed additives were often found locally in the area of the study being carried out. An alternative feeding solution allows for cheaper and better results for the environment as well as the animals.

The many secondary plant metabolites in forages, plant marcs, essential oils and extracts play a part in the anti-methanogenic effect of certain plant additives. From the studies, most often polyphenols such as condensed tannins and saponins showed significant results in greatly reducing methane gas emission. This is due to the inhibitory effect these phytochemicals have on methanogens, protozoa and organisms that produce hydrogen in the rumen microbiome.

Grape pomace and marc allow for all parts of the fruit to be used and the waste disposal to be reduced. In developing countries, this can have a huge impact on farmers making available cheaper alternatives as well as mitigating their overall contribution to methane production. Alfalfa silage is one of the most common silages in ruminant production due to its high protein content. However, most alfalfa protein is hydrolysed into nonprotein nitrogen (NPN) during ensiling which cannot be effectively used by ruminants and is excreted as urinary nitrogen, resulting in protein loss and environmental pollution. Thus, by using grape pomace as alternative feedstuff, it makes use of a waste by-product as well as is greatly beneficial to the ruminants[34]. The large amounts of tannins in grape pomace improve the efficiency of feed by decreasing methane production, alters nitrogen metabolism and improves rumen fermentation. By supplementing grape pomace in the basal diet of ruminants, farmers can take advantage of unwanted waste by-products as animal feed. However more in vivo trials should take place to confirm these preliminary findings.

With decrease in dry matter intake, supplemented seaweed has high CH4 mitigating effects of up to 60% [59]. It is easily available in certain regions that it is native to and its CH4 reduction results are undeniable and can be of great use for a cleaner future in the livestock industry.

There was significant mitigation of CH4 up to 18% and 16% by the supplementation of Gryllus bimaculatus and Bombyx mori. These edible insects were also rich in protein and their essential amino acids and amino acid composition was similar to that of soybean meal. There is a possible future in possibly replacing 25% of the soybean meal used in the diet for these edible insect species. Before this can take place, more in vivo trials have to be carried out to confirm if these findings are similar to that of live ruminants.

With authorised feed additives such as Mootral and Bovaer on the market, I believe it is possible to take the step towards a less poluting ruminant production process. However, more research should be done in vivo for Mootral to be utilised for longer periods due to its transient effect seen in the study earlier. It was also seen to increase the concentration of pyridoxine which could potentially be of great additional benefit when supplementing this additive.

3-NOP as a feed additive has a vast amount of in vivo research done which allows for the feed additive to be reliable, safe, and readily usable. It demonstrated that it was able to shift microbial activity away from methanogenic species found in the rumen microbiota by moving fermentation towards bacteria species that benefit from hydrogen which is in excess.

Many forage and plant marc feed additives have only crossed the in vitro process therefore further in vivo experiments must take place before their further utilisation can happen. When in vivo studies are carried out, the feed intake, nutrient digestibility, milk yield and lastly CH_4 emissions should be taken into consideration. It is vital to take these production parameters into account for the feed additive to be considered efficient and marketable in the industry.

There are not many studies done on small ruminants, of which there's a huge market for. Although the methane emission contributed by sheep and goats is far less in comparison to dairy cattle, it should still be considered in the grand scheme of this.

7. Summary

From the information hereby presented, feeding ruminants valuable feed additives such as Asparagopsis taxiformis, Acacia mearnsii extract, oats, Indian Gooseberry and 3-nitrooxypropanol with clear in vivo results show great potential in significantly reducing methane emissions in various ruminant species and age. As seen in the feed additives mentioned here, most contain secondary plant metabolites that have a huge effect on mitigating CH_4 emissions. By doing so livestock production processes can reach a greener and cleaner approach in the industry. More in vivo studies should be carried out to confirm preliminary findings of the in vitro studies allowing for more opportunities to be utilised by everyone.

8. References

[1] C. Matthews, F. Crispie, E. Lewis, M. Reid, P. W. O'Toole, and P. D. Cotter, 'The rumen microbiome: a crucial consideration when optimising milk and meat production and nitrogen utilisation efficiency', *Gut Microbes*, vol. 10, no. 2, pp. 115–132, Sep. 2018, doi: 10.1080/19490976.2018.1505176.

[2] 'Understanding the Ruminant Animal Digestive System | Mississippi State University Extension Service'. Accessed: Feb. 11, 2024. [Online]. Available: https://extension.msstate.edu/publications/understanding-the-ruminant-animal-digestive-system

[3] K. A. Johnson and D. E. Johnson, 'Methane emissions from cattle', *J. Anim. Sci.*, vol. 73, no. 8, pp. 2483–2492, Aug. 1995, doi: 10.2527/1995.7382483x.

[4] 'Removing methane from the atmosphere | Stanford Doerr School of Sustainability'. Accessed: Feb. 11, 2024. [Online]. Available: https://sustainability.stanford.edu/news/removing-methane-atmosphere

[5] J. C. Ku-Vera *et al.*, 'Role of secondary plant metabolites on enteric methane mitigation in ruminants.', *Front. Vet. Sci.*, vol. 6, no. August, 2020, doi: 10.3389/fvets.2020.00584.

[6] T. J. Lawton and A. C. Rosenzweig, 'Biocatalysts for methane conversion: Big progress on breaking a small substrate', *Curr. Opin. Chem. Biol.*, vol. 35, p. 142, Dec. 2016, doi: 10.1016/j.cbpa.2016.10.001.

[7] 'Bovaer®', @anh. Accessed: Mar. 08, 2024. [Online]. Available: https://www.dsm.com/anh/products-and-services/products/methane-inhibitors/bovaer.html

[8] 'Ruminant Nutrition - an overview | ScienceDirect Topics'. Accessed: Mar. 06, 2024. [Online]. Available: https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/ruminant-nutrition

[9] Wang MuSen *et al.*, 'Ensiling characteristics, in vitro rumen fermentation profile, methane emission and archaeal and protozoal community of silage prepared with alfalfa, sainfoin and their mixture.', *Anim. Feed Sci. Technol.*, vol. 284, 2022, doi: 10.1016/j.anifeedsci.2021.115154.

[10] S. U. Wetzels *et al.*, 'The application of rumen simulation technique (RUSITEC) for studying dynamics of the bacterial community and metabolome in rumen fluid and the effects of a challenge with Clostridium perfringens', *PLoS ONE*, vol. 13, no. 2, p. e0192256, Feb. 2018, doi: 10.1371/journal.pone.0192256.

[11] 'Fibrobacter Succinogenes - an overview | ScienceDirect Topics'. Accessed: Mar. 06, 2024. [Online]. Available: https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/fibrobacter-succinogenes

[12] H. Huang *et al.*, 'The effect of ensiled paulownia leaves in a high-forage diet on ruminal fermentation, methane production, fatty acid composition, and milk production performance of dairy cows', *J. Anim. Sci. Biotechnol.*, vol. 13, no. 1, p. 104, Aug. 2022, doi: 10.1186/s40104-022-00745-9.

[13] T. M. Wardiny, T. E. A. Sinar, and A. Jayanegara, 'Evaluation of noni (Morinda citrifolia) leaves and fruits on methane emission and rumen fermentation parameters in vitro.', *IOP Conf. Ser. Earth Environ. Sci.*, vol. 788, 2021, doi: 10.1088/1755-1315/788/1/012030.

[14] S. J. Lee *et al.*, 'Effects of Olive (Olea europaea L.) Leaves with Antioxidant and Antimicrobial Activities on In Vitro Ruminal Fermentation and Methane Emission', *Animals*, vol. 11, no. 7, Art. no. 7, Jul. 2021, doi: 10.3390/ani11072008.

[15] M. Ramin, P. Fant, and P. Huhtanen, 'The effects of gradual replacement of barley with oats on enteric methane emissions, rumen fermentation, milk production, and energy utilization in dairy cows.', *J. Dairy Sci.*, vol. 104, no. 5, pp. 5617–5630, 2021, doi: 10.3168/jds.2020-19644.

[16] M. Terranova, L. Eggerschwiler, S. Ortmann, M. Clauss, M. Kreuzer, and A. Schwarm, 'Increasing the proportion of hazel leaves in the diet of dairy cows reduced methane yield and excretion of nitrogen in volatile form, but not milk yield.', *Anim. Feed Sci. Technol.*, vol. 276, 2021, doi: 10.1016/j.anifeedsci.2020.114790.

[17] C. J. L. du Toit, W. A. van Niekerk, H. H. Meissner, L. J. Erasmus, and R. J. Coertze, 'Methane emissions from sheep fed Eragrostis curvula hay substituted with Lespedeza cuneata.', *Anim. Prod. Sci.*, vol. 60, no. 15, pp. 1777–1784, 2020, doi: 10.1071/AN19257.

[18] K. L. Xie, Z. F. Wang, Y. R. Guo, C. Zhang, W. H. Zhu, and F. J. Hou, ‘Gentiana straminea supplementation improves feed intake, nitrogen and energy utilization, and methane emission of Simmental calves in Northwest China.’, *Anim. Biosci.*, vol. 35, no. 6, pp. 838–846, 2022, doi: 10.5713/ab.21.0263.
[19] P. J. Moate *et al.*, ‘Effects of Feeding either Red or White Grape Marc on Milk Production and Methane Emissions from Early-Lactation Dairy Cows’, *Animals*, vol. 10, no. 6, Art. no. 6, Jun. 2020, doi: 10.3390/ani10060976.
[20] X. Zhang *et al.*, ‘Microbial mechanisms of using feruloyl esterase-producing *Lactobacillus plantarum* A1 and grape pomace to improve fermentation quality and mitigate ruminal methane emission of ensiled alfalfa for cleaner animal production’, *J. Environ. Manage.*, vol. 308, p. 114637, Apr. 2022, doi: 10.1016/j.jenvman.2022.114637.
[21] Ankita Singla, J. S. Hundal, A. K. Patra, Manju Wadhwa, Veena Nagarajappa, and Puneet Malhotra, ‘Effect of dietary supplementation of Emblica officinalis fruit pomace on methane emission, ruminal fermentation, nutrient utilization, and milk production performance in buffaloes.’, *Environ. Sci. Pollut. Res.*, vol. 28, no. 14, pp. 18120–18133, 2021, doi: 10.1007/s11356-020-12008-z.
[22] M. S. Ala *et al.*, ‘Potential of walnut (Juglans regia) leave ethanolic extract to modify ruminal fermentation, microbial populations and mitigate methane emission’, *Anim. Prod. Sci.*, vol. 60, no. 9, pp. 1189–1200, Apr. 2020, doi: 10.1071/AN19241.
[23] Lee ShinJa, Shin NyeonHak, Jeong JinSuk, Kim EunTae, Lee SuKyoung, and Lee SungSill, ‘Effect of Rhodophyta extracts on in vitro ruminal fermentation characteristics, methanogenesis and microbial populations.’, *Asian-Australas. J. Anim. Sci.*, vol. 31, no. 1, pp. 54–62, 2018, doi: 10.5713/ajas.17.0620.
[24] T. M. Denninger *et al.*, ‘Immediate effect of Acacia mearnsii tannins on methane emissions and milk fatty acid profiles of dairy cows.’, *Anim. Feed Sci. Technol.*, vol. 261, 2020, doi: 10.1016/j.anifeedsci.2019.114388.
[25] F. Hassanat and C. Benchaar, ‘Corn silage-based diet supplemented with increasing amounts of linseed oil: Effects on methane production, rumen fermentation, nutrient digestibility, nitrogen utilization, and milk production of dairy cows’, *J. Dairy Sci.*, vol. 104, no. 5, pp. 5375–5390, May 2021, doi: 10.3168/jds.2020-18853.
[26] A. L. Shreck, J. M. Zeltwanger, E. A. Bailey, J. S. Jennings, B. E. Meyer, and N. A. Cole, ‘Effects of protein supplementation to steers consuming low-quality forages on greenhouse gas emissions.’, *J. Anim. Sci.*, vol. 99, no. 7, 2021, doi: 10.1093/jas/skab147.
[27] N. A. Cole *et al.*, ‘Effects of diet quality on energy metabolism and methane production by beef steers fed a warm-season grass-based hay diet.’, *Appl. Anim. Sci.*, vol. 36, no. 5, pp. 652–667, 2020, doi: 10.15232/aas.2020-02025.
[28] E. del Jesus Flores-Santiago *et al.*, ‘Reduction of enteric methane emissions in heifers fed tropical grass-based rations supplemented with palm oil.’, *Fermentation*, vol. 8, no. 8, 2022, doi: 10.3390/fermentation8080349.
[29] Lee ShinJa *et al.*, ‘Impact of Ecklonia stolonifera extract on in vitro ruminal fermentation characteristics, methanogenesis, and microbial populations.’, *Asian-Australas. J. Anim. Sci.*, vol. 32, no. 12, pp. 1864–1872, 2019.
[30] Y. R. Kim *et al.*, ‘Rumen methane abatement by phlorotannin derivatives (phlorofucofuroeckol-a, dieckol, and 8, 8’-bieckol) and its relationship with the hydroxyl group and ether linkage.’, *Anim. Feed Sci. Technol.*, vol. 293, 2022, doi: 10.1016/j.anifeedsci.2022.115468.
[31] H. A. Stefenoni *et al.*, ‘Effects of the macroalga Asparagopsis taxiformis and oregano leaves on methane emission, rumen fermentation, and lactational performance of dairy cows.’, *J. Dairy Sci.*, vol. 104, no. 4, pp. 4157–4173, 2021, doi: 10.3168/jds.2020-19686.
[32] B. M. Roque, J. K. Salwen, R. Kinley, and E. Kebreab, ‘Inclusion of Asparagopsis armata in lactating dairy cows’ diet reduces enteric methane emission by over 50 percent’, *J. Clean. Prod.*, vol. 234, pp. 132–138, Oct. 2019, doi: 10.1016/j.jclepro.2019.06.193.
[33] Y.-L. Wang *et al.*, ‘The Effect of Different Lactic Acid Bacteria Inoculants on Silage Quality, Phenolic Acid Profiles, Bacterial Community and In Vitro Rumen Fermentation Characteristic of Whole Corn Silage’, *Fermentation*, vol. 8, no. 6, Art. no. 6, Jun. 2022, doi: 10.3390/fermentation8060285.

[34] Zhang Xia *et al.*, ‘Microbial mechanisms of using feruloyl esterase-producing Lactobacillus plantarum A1 and grape pomace to improve fermentation quality and mitigate ruminal methane emission of ensiled alfalfa for cleaner animal production.’, *J. Environ. Manage.*, vol. 308, 2022, doi: 10.1016/j.jenvman.2022.114637.
[35] Pang Jie *et al.*, ‘A novel identified Pseudomonas aeruginosa, which exhibited nitrate- and nitrite-dependent methane oxidation abilities, could alleviate the disadvantages caused by nitrate supplementation in rumen fluid fermentation.’, *Microb. Biotechnol.*, vol. 14, no. 4, pp. 1397–1408, 2020, doi: 10.1111/1751-7915.13726.
[36] X. Zhao *et al.*, ‘High-potency white-rot fungal strains and duration of fermentation to optimize corn straw as ruminant feed’, *Bioresour. Technol.*, vol. 312, p. 123512, Sep. 2020, doi: 10.1016/j.biortech.2020.123512.
[37] E. Ahmed, N. Fukuma, M. Hanada, and T. Nishida, ‘Insects as Novel Ruminant Feed and a Potential Mitigation Strategy for Methane Emissions’, *Animals*, vol. 11, no. 9, Art. no. 9, Sep. 2021, doi: 10.3390/ani11092648.
[38] R. A. P. Purba, S. Paengkoum, C. Yuangklang, and P. Paengkoum, ‘Flavonoids and their aromatic derivatives in Piper betle powder promote in vitro methane mitigation in a variety of diets.’, *Ciênc. E Agrotecnologia*, vol. 44, 2020, doi: 10.1590/1413-7054202044012420.
[39] G. Thirumalaisamy, P. K. Malik, A. P. Kolte, S. Trivedi, A. Dhali, and R. Bhatta, ‘Effect of silkworm (Bombyx mori) pupae oil supplementation on enteric methane emission and methanogens diversity in sheep.’, *Anim. Biotechnol.*, vol. 33, no. 1, pp. 128–140, 2022, doi: 10.1080/10495398.2020.1781147.
[40] Tong JinJin, Zhang Hua, Wang Jia, Liu Yun, Mao ShengYong, and Jiang LinShu, ‘Effects of different molecular weights of chitosan on methane production and bacterial community structure in vitro.’, *J. Integr. Agric.*, vol. 19, no. 6, pp. 1644–1655, 2020, doi: 10.1016/S2095-3119(20)63174-4.
[41] ‘Mootral | Enterix™’. Accessed: Mar. 08, 2024. [Online]. Available: https://mootral.com/solutions/enterix
[42] ‘Evaluating the effects of high-oil rapeseed cake or natural additives on methane emissions and performance of dairy cows - ScienceDirect’. Accessed: Sep. 17, 2023. [Online]. Available: https://www.sciencedirect.com/science/article/pii/S0022030221010146
[43] J. Brede, M. Peukert, B. Egert, G. Breves, and M. Brede, ‘Long-Term Mootral Application Impacts Methane Production and the Microbial Community in the Rumen Simulation Technique System’, *Front. Microbiol.*, vol. 12, 2021, Accessed: Sep. 17, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fmicb.2021.691502
[44] ‘Minimizing methane from cattle | DSM’, @corporate. Accessed: Sep. 17, 2023. [Online]. Available: https://www.dsm.com/corporate/sustainability/our-purpose/minimizing-methane-from-cattle.html
[45] ‘Taking action on climate change, together’. Version 1. Issued at the occasion of the 7th Greenhouse Gas and Animal Agriculture Conference, Aug. 2019. [Online]. Available: https://www.dsm.com/content/dam/dsm/corporate/en_US/documents/summary-scientific-papers-3nop-booklet.pdf
[46] F. Garcia *et al.*, ‘3-Nitrooxypropanol substantially decreased enteric methane emissions of dairy cows fed true protein- or urea-containing diets.’, *Heliyon*, vol. 8, no. 6, 2022, doi: 10.1016/j.heliyon.2022.e09738.
[47] A. W. Alemu *et al.*, ‘Use of 3-nitrooxypropanol in a commercial feedlot to decrease enteric methane emissions from cattle fed a corn-based finishing diet.’, *J. Anim. Sci.*, vol. 99, no. 1, 2020, doi: 10.1093/jas/skaa394.
[48] S. van Gastelen *et al.*, ‘3-nitrooxypropanol decreases methane emissions and increases hydrogen emissions of early lactation dairy cows, with associated changes in nutrient digestibility and energy metabolism.’, *J. Dairy Sci.*, vol. 103, no. 9, pp. 8074–8093, 2020, doi: 10.3168/jds.2019-17936.
[49] A. Melgar *et al.*, ‘Dose-response effect of 3-nitrooxypropanol on enteric methane emissions in dairy cows.’, *J. Dairy Sci.*, vol. 103, no. 7, pp. 6145–6156, 2020, doi: 10.3168/jds.2019-17840.
[50] E. Cardoso-Gutierrez *et al.*, ‘Effect of tannins from tropical plants on methane production from ruminants: A systematic review’, *Vet. Anim. Sci.*, vol. 14, p. 100214, Dec. 2021, doi:

10.1016/j.vas.2021.100214.
[51] K. Jamwal, S. Bhattacharya, and S. Puri, 'Plant growth regulator mediated consequences of secondary metabolites in medicinal plants', *J. Appl. Res. Med. Aromat. Plants*, vol. 9, pp. 26–38, May 2018, doi: 10.1016/j.jarmap.2017.12.003.
[52] G. Yu, K. A. Beauchemin, and R. Dong, 'A Review of 3-Nitrooxypropanol for Enteric Methane Mitigation from Ruminant Livestock', *Anim. Open Access J. MDPI*, vol. 11, no. 12, p. 3540, Dec. 2021, doi: 10.3390/ani11123540.
[53] I. M. L. D. Storm, A. L. F. Hellwing, N. I. Nielsen, and J. Madsen, 'Methods for Measuring and Estimating Methane Emission from Ruminants', *Anim. Open Access J. MDPI*, vol. 2, no. 2, pp. 160–183, Apr. 2012, doi: 10.3390/ani2020160.
[54] C. G. Brooke *et al.*, 'Methane Reduction Potential of Two Pacific Coast Macroalgae During in vitro Ruminant Fermentation', *Front. Mar. Sci.*, vol. 7, Jul. 2020, doi: 10.3389/fmars.2020.00561.
[55] 'GreenFeed Pasture System', C-Lock Inc. Accessed: Mar. 07, 2024. [Online]. Available: https://www.c-lockinc.com/products/emissions-monitoring/greenfeed-pasture-system
[56] 'NJV spectral lab', SLU.SE. Accessed: Mar. 08, 2024. [Online]. Available: https://www.slu.se/en/departments/crop-production-ecology/resurser/new-robacksdalens--field-research-station/spektrala-tekniker/
[57] Y. Rochette, A. Jonker, P. Moate, A. Vanlierde, and C. Martin, 'Sulphur hexafluoride (SF_6) tracer technique', https://books.publisso.de/en/publisso_gold/publishing/books/overview/53/196. Accessed: Mar. 08, 2024. [Online]. Available: https://books.publisso.de/en/publisso_gold/publishing/books/overview/53/196
[58] P. J. Moate *et al.*, 'Measurement of Enteric Methane Emissions by the SF6 Technique Is Not Affected by Ambient Weather Conditions', *Anim. Open Access J. MDPI*, vol. 11, no. 2, p. 528, Feb. 2021, doi: 10.3390/ani11020528.
[59] 'Effects of Red Seaweed on Milk Production and Methane Emissions', College of Life Sciences and Agriculture. Accessed: Mar. 08, 2024. [Online]. Available: https://colsa.unh.edu/resource/effects-red-seaweed-milk-production-methane-emissions

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