COMPARATIVE EVALUATION OF COMBINATION OF THE METHANOGENIC INHIBITORS ON ENTERIC METHANE EMISSION AND NUTRIENT DIGESTIBILITY IN SAHIWAL AND GIR CALVES

THESIS SUBMITTED TO THE

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL

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IN PARTIAL FULFILMENT OF THE REQUIREMENT

FOR THE DEGREE OF

MASTER OF VETERINARY SCIENCE
IN
ANIMAL NUTRITION
BY
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(B.V.Sc &A.H)

DIVISION OF ANIMAL NUTRITION

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE

(DEEMED UNIVERSITY)

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IN
ANIMAL NUTRITION

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Dated: **13, December 2021**

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ABBREVIATIONS

%	Percent
@	At the rate
A: P	Acetate to Propionate ratio
ADF	Acid detergent fiber
ADG	Average daily gain
ADICP	Acid detergent insoluble crude protein
ADIN	Acid detergent insoluble nitrogen
ADL	Acid detergent lignin
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AOAC	Association of Analytical Chemists
AST	Aspartate aminotransferase
BC	Buffering capacity
BW	Bodyweight
℃	Degree Celsius
CF	Crude Fiber
CFU	colony forming units
CH_4	Methane
cm	Centimeter
CO_2	Carbon Dioxide
CP	Crude protein
CTAB	Cetyltrimethylammonium ammonium bromide
$CuSo_4$	Copper Sulphate

D	Day
DCP	Digestible Crude Protein
DE	Digestible Energy
DM	Dry matter
DORB	De-oiled rice bran
EDTA	Ethylene diamine tetraacetate
EE	Ether Extract
FCR	Feed conversion ratio
FID	Flame Ionization Detector
Fig	Figure
g	Gram
GC	Gas Chromatography
GE	Gross Energy
GNC	Groundnut cake
GP	Gas Production
h	hour(s)
H_2	Hydrogen gas
H_2O	Water
H_2SO_4	Sulphuric acid
HC	Hemicellulose
He	Heterofermentative
Ho	Homofermentative
ICAR	Indian Council of Agricultural Research
IVFA	Individual Volatile Fatty Acids

IVGP	*In vitro* Gas Production
IVMP	*In vitro* Methane Production
IVNDFD	*In vitro* Neutral Detergent Fiber Digestibility
IVTDMD	*In vitro* True Dry Matter Digestibility
IVTOMD	*In vitro* True Organic Matter Digestibility
kcal	Kilocalorie
kg	Kilogram
L	Liter
LA	Lactic acid
MBP	Microbial biomass production
Mcal	Mega calorie
MCP	Microbial crude protein
ME	Metabolizable Energy
Mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimole
mM/L	Millimole per liter
N	Nitrogen
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NDF	Neutral Detergent Fiber
NDRI	National Dairy Research Institute

NDS	Neutral Detergent Solution
NFE	Nitrogen Free Extract
NH_3	Ammonia
NH_3-N	Ammonia-N
No	Number
N_2O	Nitrous Oxide
NPN	Non-Protein Nitrogen
NRC	National Research Council
OM	Organic Matter
OMD	Organic Matter Digestibility
OMI	Organic Matter Intake
PDA	Potato Dextrose Agar
PF	Partition factor
SCFA	Short-chain fatty acid
Sec	Second
sp.	Species
SRL	Strained Rumen Liquor
TA	Total ash
TCA	Trichloroacetic Acid
TDN	Total Digestible Nutrients
TG	Total Gas
TVFA	Total Volatile Fatty Acid
U/L	unit per liter
VFA	Volatile Fatty Acid

$W^{0.75}$	Metabolic body weight
WB	Wheat bran
WSC	Water-soluble carbohydrate
µg	Microgram
µl	Microliter

COMPARATIVE EVALUATION OF COMBINATION OF THE METHANOGENIC INHIBITORS ON ENTERIC METHANE EMISSION AND NUTRIENT DIGESTIBILITY IN SAHIWAL AND GIR CALVES

ABSTRACT

The present study was undertaken to estimate the comparative evaluation of the combination of methanogenic inhibitors on enteric methane emission, nutrient digestibility, and rumen fermentation patterns in Sahiwal and Gir calves. The study consisted of a total of 12 healthy, 6 Sahiwal calves and 6 Gir calves. The animals were divided into 4 groups of 3 animals in each, based on their average body weight. In the control groups of both the species the animals fed diet as per ICAR (2013) and in treatment groups animals were fed with control diet + combination of methane inhibitors (nitrate, linseed oil, and anthraquinone). The design of the experiment was switch over consisting of period 1 and period 2 each of 60 days. During the last week of each period of the experiment, a digestibility trial of seven days was conducted with a total collection of faeces, residue, and feed to study the digestibility of various nutrients. The methane was estimated by using the SF_6 tracer technique by taking five successful collections and the rumen fermentation pattern was studied by collecting rumen liquor at the end of each period.

There was no significant difference in DMI and nutrient digestibility among control and treatment groups of both Sahiwal and Gir calves, this implied that supplementation of methane inhibitors (nitrate, linseed oil, and anthraquinone) did not affect the intake and digestibility of the nutrients.

In the case of rumen fermentation pattern, there was no significant change in acetate, but an increase in propionate and decrease in butyrate values was observed in treatment groups of both breeds when compared with that of control groups. Whereas Total-N and NH_3N increased but pH of rumen liquor decreased. CH_4 emission (g/d) was tended to be lower in treatment groups fed with methane inhibitors (nitrate, linseed oil, and anthraquinone combination) (51.90±2.88 in Sahiwal calves and 61.38±1.43 in Gir calves) as compared to groups fed with control diet (65.39±2.70 in Sahiwal, 74.55±1.01 in Gir). No significant ($P>0.05$) changes were seen in CH_4 g/kg DMI, DDMI, OMI, DOMI, NDFI, DNDFI, ADFI, DADFI. The values of methane emissionper kg DMI and DDMI ranged between 13-17g and 21 to 27 g respectively in the four groups. Thevalues per kg OMI and DOMI ranged between 14-19 and 23-30g respectively. Intake of energy (GEI, DEI, MEI) was not significantly different in groups fed with control diet when compared

with treatment groups fed with methane inhibitors because there was no effect on intake as well as digestibility of the feeds in all the four groups.

CH_4 energy (MJ/d) was significantly more in control groups (Sahiwal (C) 3.60±0.14, Gir (C) 4.10±0.05) as compared to their treatment groups (Sahiwal (T) 2.85±0.14, Gir (T) 3.38±0.07). CH_4 energy loss as DEI (%) was significantly ($P<0.05$) higher in control groups (Sahiwal (C) 8.08±0.65, Gir (C) 8.63±0.53,) when compared with treatment groups supplemented with methane inhibitors ((Sahiwal (T) 6.44±0.36, Gir (T) 7.39±0.41). There was no significant difference in methane energy loss as % GEI, but the decreasing trend was observed for methane energy loss as % MEI in treatment groups when compared with control.

Thus, this study indicated that the supplementation of a combination of methane inhibitors (nitrate, linseed oil, and anthraquinone) has not affected nutrient intake and nutrients digestibility in both the breeds, Sahiwal and Gir calves. It has caused an increase in propionate and nitrogen in the rumen while methane emission decreased.

सार

वर्तमान अध्ययन साहीवाल और गिर बछड़ों में एंटेरिक मीथेन उत्सर्जन, पोषक तत्वों की पाचन क्षमता और रुमेन किण्वन पैटर्न पर मेथेनोजेनिक अवरोधकों के संयोजन के तुलनात्मक मूल्यांकन का अनुमान लगाने के लिए किया गया था। अध्ययन में कुल 12 स्वस्थ 6 साहीवाल बछड़े और 6 गिर बछड़े शामिल हैं। जानवरों को उनके औसत शरीर के वजन के आधार पर प्रत्येक में 3 जानवरों के 4 समूहों में विभाजित किया गया था। दोनों प्रजातियों के नियंत्रण समूहों में जानवरों को आईसीएआर 2013 के अनुसार आहार दिया गया और उपचार समूहों में जानवरों को नियंत्रण आहार + मीथेन अवरोधकों (नाइट्रेट, अलसी का तेल और एन्थ्राक्विनोन) के संयोजन के साथ खिलाया गया। प्रयोग के डिजाइन को 60 दिनों की अवधि 1 और अवधि 2 से युक्त किया गया था। प्रयोग की प्रत्येक अवधि के अंतिम सप्ताह के दौरान, मल, अवशेष और फ़ीड के कुल संग्रह के साथ सात दिनों का पाचनशक्ति परीक्षण किया गया था। विभिन्न पोषक तत्वों की पाचनशक्ति का अध्ययन करने के लिए। प्रत्येक अवधि के अंत में रुमेन शराब एकत्र करके अध्ययन किए गए पांच सफल संग्रह और रुमेन किण्वन पैटर्न लेकर एसएफ 6 ट्रेसर तकनीक का उपयोग करके मीथेन का अनुमान लगाया गया था।

साहीवाल और गिर दोनों नस्लों के नियंत्रण और उपचार समूहों के बीच डीएमआई और पोषक तत्वों की पाचनशक्ति का कोई महत्वपूर्ण अंतर नहीं था, इसका मतलब है कि मीथेन अवरोधकों (नाइट्रेट, अलसी का तेल, और एन्थ्राक्विनोन) का पूरक पोषक तत्वों के सेवन को प्रभावित नहीं करता है।

रूमेन किण्वन पैटर्न के मामले में एसीटेट में कोई महत्वपूर्ण परिवर्तन नहीं हुआ था, लेकिन नियंत्रण समूहों की तुलना में साहीवाल और गिर के उपचार समूहों में प्रोपियोनेट में वृद्धि और ब्यूटायरेट मूल्यों में कमी देखी गई थी। जबकि Total-N2 और NH3N2 में वृद्धि हुई है, लेकिन रुमेन शराब का pH कम हो गया है। CH4 उत्सर्जन (g/d) को मीथेन इनहिबिटर (नाइट्रेट, अलसी का तेल, और एन्थ्राक्विनोन संयोजन) (51.90±2.88) के साथ खिलाए गए उपचार समूहों में कम किया गया था (साहिवाल में 51.90±2.88) बछड़ों, 61.38 ± 1.43 गिर बछड़ों में) की तुलना में नियंत्रण आहार (65.39 ± 2.70 साहीवाल में, 74.55 ± 1.01 गिर में) से खिलाए गए समूहों की तुलना में। CH4 g/kg DMI, DDMI, OMI, DOMI, NDFI, DNDFI, ADFI, DADFI में कोई महत्वपूर्ण (P > 0.05) परिवर्तन नहीं देखा गया। चार समूहों में मीथेन उत्सर्जन प्रति किग्रा डीएमआई और डीडीएमआई क्रमशः 13-17 ग्राम और 21 से 27 ग्राम के बीच था। मूल्य प्रति किग्रा ओएमआई और डोमी क्रमशः 14-19

और 23-30 ग्राम के बीच थे। मीथेन अवरोधकों के साथ खिलाए गए उपचार समूहों की तुलना में नियंत्रण आहार से खिलाए गए समूहों में ऊर्जा का सेवन (जीईआई, डीईआई, एमईआई) काफी भिन्न नहीं था क्योंकि सभी चार समूहों में सेवन के साथ-साथ फ़ीड की पाचनशक्ति पर कोई प्रभाव नहीं पड़ा।

सीएच4 ऊर्जा (एमजे/डी) उनके उपचार समूहों की तुलना में नियंत्रण समूहों (साहिवाल (सी) 3.60±0.14, गिर (सी) 4.10±0.05) में काफी अधिक है (साहिवाल (टी) 2.85±0.14, गिर (टी) 3.38 ±0.07)। डीईआई (%) के रूप में सीएच 4 ऊर्जा हानि काफी (पी <0.05) नियंत्रण समूहों में अधिक थी (साहिवाल (सी) 8.08 ± 0.65, गिर (सी) 8.63 ± 0.53, जब मीथेन इनहिबिटर (साहिवाल (टी) के पूरक उपचार समूहों के साथ तुलना की जाती है। 6.44 ± 0.36, गिर (टी) 7.39 ± 0.41।% जीईआई के रूप में मीथेन ऊर्जा हानि में कोई महत्वपूर्ण अंतर नहीं है, लेकिन नियंत्रण के साथ तुलना करने पर उपचार समूहों में% एमईआई के रूप में मीथेन ऊर्जा हानि के लिए घटती प्रवृत्ति देखी गई थी।

इस प्रकार, इस अध्ययन ने संकेत दिया कि मीथेन अवरोधकों (नाइट्रेट, अलसी का तेल, और एन्थ्राक्विनोन) के संयोजन के पूरक ने पोषक तत्वों के सेवन और पोषक तत्वों की पाचन क्षमता को प्रभावित किए बिना दोनों नस्लों, साहीवाल और गिर बछड़ों के उपचार समूहों में मीथेन उत्सर्जन को महत्वपूर्ण रूप से कम नहीं किया है। इससे रूमेन में प्रोपियोनेट और नाइट्रोजन में वृद्धि हुई है।

CHAPTER - 1

INTRODUCTION

Global climate change is primarily caused by greenhouse gas (GHG) emissions which result in the warming of the atmosphere. Methane is a greenhouse gas (GHG) with a global warming potential 23-fold that of carbon dioxide (Samal *et al.,* 2016) and makes up 16% of total global GHG emissions. It is probably the second most important gas after CO_2 contributing to global warming (Van Nevel and Demeyer, 1996). Around 2-15% of gross energy intake by the ruminant animals is lost as methane so, it's important to reduce energy lost as methane, which favours both animals as well as the environment.

The total bovine population (Cattle, Buffalo, Mithun, and Yak) was 302.79 Million in 2019 which showed an increase of 1.0% over the previous census. Production of enormous amounts of methane is attributed to ruminant digestive processes specifically in their rumen, which is undesirable. The rumen is a unique home to a large number of ciliated protozoa, anaerobic bacteria, anaerobic fungi, and archaea which enables them to bio-convert poor quality lignocellulolytic feed into valuable animal products like milk and meat. In the rumen, the feed taken by the animals is fermented by an interactive concerted activity of bacteria, protozoa, fungi, archaea, and bacteriophages as an outcome of which the polysaccharides of feed are transformed into volatile fatty acids and microbial protein, the two main sources of energy and protein for host animals. But during feed fermentation in the rumen, CO_2 and H_2 gases are produced as by-products and rumen methanogenic archaea reduce CO_2 into methane by utilizing H_2 which gets eructed out through the mouth. 500-600 litres of H_2 are produced daily in adult buffaloes or cattle, but due to methanogenesis, it never accumulates in the gaseous phase of the rumen. In the rumen, methanogenesis is an essential metabolic process to maintain a low H_2 pressure but wasteful as 2-12% dietary energy (Johnson *et al.*,1993) gets wasted in the form of methane which reduces the potential conversion of dietary energy into metabolizable energy thus reducing feed utilization efficiency of the animals. Ruminants produce enormous amounts of methane accounting for about 81% of GHG from the livestock sector, 90% of which results from rumen microbial methanogenesis (Hristov *et al.,* 2015). This necessitates finding innovations and strategies to reduce the ruminant CH_4 production in the perspective of global warming and enhancing animal productivity.

The control of methanogenesis can be achieved by developing an alternative hydrogen sink to divert hydrogen away from methanogenesis (Joblin *et al.*,1999). The addition of alternate electron acceptors to the rumen seems to be a logical means of reducing methane emission. The reduction of nitrate (ΔG=-163 KJ mol) and sulphate (ΔG=152 KJ mol) is thermodynamically favoured over carbon dioxide reduction (Δ G =130 KJ/mol), which provides nitrate and sulphate-reducing bacteria a competitive advantage over methanogens as hydrogen sink (Oremland *et al.,*1988).

The inclusion of vegetable oil or lipid is another most promising technique for reducing methane emission from ruminants (Beauchemin *et al.,* 2008). Linseed fatty acids offer a promising dietary means to suppress ruminal methanogenesis.

Anthraquinone (9, 10- dioxo anthracene) is a naturally occurring aromatic organic compound found within some plants. The Rhubarb compounds 9,10-Anthracenedione, 1,8 dihydroxy-3-methyl (-6.92 kcal/mol); phthalic acid isobutyl octadecyl ester (-5.26 kcal/mol); and di iso-octyl phthalate (-5.61 kcal/mol) have better specificity towards the methyl-coenzyme M reductase binding site and could be a potent methane inhibitor (Arokiyaraj et al.,2019). Hence the combination of all three was tested in this study with the following objective.

Objective

To compare nutrient digestibility, rumen fermentation patterns, and methane emission in Sahiwal and Gir calves fed on a combination of Methane inhibitors (nitrate, linseed oil, and anthraquinone).

CHAPTER- 2

REVIEW OF LITERATURE

2.1 METHANE PRODUCTION BY RUMINANTS

The livestock sector is one of the largest sources of methane emission, producing to the tune of 80-115 million tons per year, which is equivalent to 15-20% of the total anthropogenic methane (Gerber *et al.*, 2013). Methane production by enteric fermentation in the rumen, besides contributing to the emission of greenhouse gas into the environment, accounts for approximately 2-12% of the gross energy loss to the animal (Johnson and Johnson 1995). About 85% of the methane is produced through exhalation when the animals masticate their feeds to digest it. However, methanogenesis in the rumen is an important way for electrons generated during fermentation to be disposed of, and inhibiting methanogens directly may result in hydrogen build-up, which could slow rumen fermentation. Thus, it is desirable to divert the reducing equivalents in reactions other than methanogenesis, such as acetate, from carbon dioxide and hydrogen, and selective stimulation of fermentation for increased synthesis of propionate. In developing countries, the livestock sector is the backbone of the rural economy, but the animalsin this part of the world are less productive, being reared on typically crop residue-based feed resources. It is, therefore, said that such a production system results in more methane emission per unit animal production than that of the high-yielding animals of the developed world. Singhal*et al.* (2005) observed that crossbred cattle, indigenous cattle, buffalo, goats, and sheep contributed 4.6, 48.5, 39, 4.7, and 1.8 percent of methane emission by enteric fermentation, respectively.

2.2 METHANOGENESIS AND EFFECTS OF ANTI-METHANOGENIC COMPOUNDS ON RUMEN METHANOGENS

Methanogens use only a small number of substrates and convert them into methane by three pathways viz., CO_2 reduction pathway, a pathway involving methyl-containing compounds, and acetolactic pathway (Zinder, 1993). The CO_2 reduction pathway (reduction of CO_2 with electrons from H_2 or sometimes formate) is the major pathway as most of the methanogens use it to produce methane.

Numerous methane-reducing strategies have been discovered, including interventions of animal management, dietary composition, rumen fermentation, and methanogens (Knapp *et al.*, 2014). The most effective technique among these mitigating approaches is to stop methanogens from increasing their metabolic activity. Another method is to change the rumen microbiota so that fermentation shifts away from H_2 generation and toward lower VFA production (e.g., propionate).

2.2.1 COENZYME M ANALOGS

The last stage of all methanogenesis processes is mediated by Methyl-CoM reductase (Mcr), and CoM (2-mercapto ethane sulfonic acid) is an essential cofactor that serves as the methyl group carrier. Methyl-CoM is reduced to methane by MCR. All known methanogens have CoM, but not other archaea or bacteria (Liu *et al.*, 2008). Several halogenated sulfonated compounds, including as 2-bromoethanesulfonate (BES), 2-chloroethanesulfonate (CES), and 3-bromopropanesulfonate (BPS), are structural analogues of CoM and can block Mcr action competitively and selectively, decreasing methane synthesis at low doses (Nollet *et al.*, 1997).

2.2.2 HALOGENATED ALIPHATIC C_1-C_2 HYDROCARBON

Chloroform, bromochloromethane (BCM), bromoform, bromodichloromethane, dibromochloromethane, carbon tetrachloride, trichloroacetamide, and trichloro-ethyl adipate are examples of halogenated aliphatic molecules with 1 or 2 carbons that might reduce ruminal methane production (Patra *et al.*, 2014). These halogenated compounds block the function of corrinoid enzymes and inhibit cobamide-dependent methyl group transfer in methanogenesis (Wood *et al.*, 1968). These halogenated compounds also competitively inhibit methane production by serving as terminal electron (e-) acceptors (Patra *et al.*, 2012).

2.2.3 NITROOXY COMPOUNDS

3-Nitrooxypropanol (3NOP) and ethyl-3NOP, two new synthetic compounds, have been shown to have specific anti-methanogenic properties (Patra *et al.*, 2017).

2.2.4 PTERIN COMPOUNDS

Pterins are structural analogues of deazaflavin (F420), a coenzyme that plays a role in two phases of the hydrogenotrophic methanogenesis pathway (Nagar-Anthal *et al.,* 1996). Therefore, pterin compounds competitively inhibit methane production.

2.2.5 PLANT SECONDARY METABOLITES

2.2.5.1 TANNINS

Tannins lower CH_4 generation by suppressing methanogens directly and indirectly by reducing fibre digestion and protozoal proliferation in the rumen. On pure cultures of methanogens, tannins isolated from Lotus pedunculatus were demonstrated to have inhibitory action. In the rumen of goats fed tannin-rich diets, Puchala *et al.* (2013) discovered suppression of methanogen populations.

2.2.5.2 FLAVONOIDS

Flavonoids have not been extensively studied concerning rumen methanogenesis (Patra *et al.,* 2012). Flavone, myricetin, naringin, rutin, quercetin, or kaempferol were reported to lower in-vitro CH4 production by 5 to 9 mL/ g DM, according to Oskoueian *et al.* (2013). Flavonoids directly inhibit methanogens and also likely act as H_2 sinks via cleavage of ring structures (e.g., catechin) and reductive dihydroxylation.

2.2.5.3 SAPONINS

The effects of saponins on rumen microbial populations, rumen fermentation, and ruminant productivity have been studied extensively and reviewed previously (Cieslak *et al.,* 2013). Saponins probably have a slightly direct effect on methanogens but are known to inhibit rumen protozoa and lowering H_2 production (Patra and Saxena, 2009).

2.2.5.4 ESSENTIAL OILS

The effects, mostly beneficial, of essential oils (EO) on rumen fermentation, microbial populations, and ruminant productivity have frequently been reviewed (Ceislak *et al.*, 2013; Calsamiglia *et al.*, 2007). Several EO compounds, either in pure form or in mixtures, are anti-methanogenic (Patra *et al.*, 2014; Khorrami *et al.*, 2015). The effects of EO on CH_4 production and methanogens are variable depending on types, dose, and diet. Overall, EOs may directly or indirectly suppress methanogens in the rumen by inhibiting protozoa and H_2 generating bacteria.

2.2.6 ALTERNATIVE HYDROGEN SINKS

2.2.6.1 NITRATE AND SULPHATE

Nitrate (NO_3^-) decreased methane production both *in vitro* and *in vivo* (Patra *et al.*, 2013). Mechanistically, nitrate reduces methane production by competing with CO as an e-acceptor, and its reduction intermediates, nitrite (NO_2^-) and nitrous oxide (N_2O), inhibit methanogens andsome H_2 producers directly (Le *et al.*, 2012). Sulphate also mitigates CH_4 production, but much less effectively than nitrate.

2.2.6.2 NITRO COMPOUNDS

A few organic nitro compounds have been evaluated for their efficacy to reduce methanogens and CH_4 production (Latham et al., 2016). These compounds can act as e- acceptors by some bacteria competing with methanogens for reducing equivalents. Nitro compounds may also inhibit methanogenesis via decreasing the activity of formate dehydrogenase, formate hydrogen lyase, and hydrogenase, which are all involved in the first step(s) of the hydrogenotrophic methanogenesis pathway, or by inhibiting e- transfer between ferredoxin and hydrogenase.

2.2.6.3 PROPIONATE AND BUTYRATE ENHANCERS

Carbohydrate fermentation intermediates include malate, fumarate, oxaloacetate, and acrylate. They can be converted to propionate or utilised in the production of amino acids and other compounds during anabolism. They can accept reducing equivalents, lowering the amount of H_2 available for CH_4 synthesis stoichiometrically. In continuous fermenters using forages as a substrate, fumarate reduced methane generation by 38 percent when added at a concentration of 3.5 g/L (Kolver *et al.,* 2004).

2.2.6.4 UNSATURATED ORGANIC ACIDS

During biohydrogenation, unsaturated fatty acids can act as hydrogen sinks, reducing methane generation. In vitro, propenoic acid (an unsaturated analogue of propionic acid), 3-butenoic acid and 2-butenoic acid (both unsaturated analogues of butyric acid), and ethyl 2 butenoate were tested as alternate e- sinks to reduce methanogenesis (Ungerfeld *et al.*, 2003).

2.2.7 INHIBITORS TO HYDROGEN-PRODUCING BACTERIA

2.2.7.1 IONOPHORES

Ionophores mainly inhibit Gram-positive bacteria, including members of class Clostridia, including Ruminococcus species that produce acetate and H^+, (Chalupa *et al.,* 1988). Ionophores can also inhibit some Gram-negative rumen bacteria, including bacteria that produce formate and H_2 (Kim *et al.,* 2016). Therefore, ionophores may decrease methane emissions by decreasingH_2 production.

2.2.7.2 BACTERIOCINS

Bacteriocins are bacteria-produced proteins or peptides that inhibit specific microbial species in the rumen and other environments. Only a few research have looked into the impact of bacteriocins on CH_4 emissions. In vitro, Bovicin HC5, a bacteriocin generated by Streptococcus spp. in the rumen, was found to decrease CH_4 by 50% (Lee *et al.,* 2002). In vitro, Nisin, a bacteriocin generated by Lactobacillus lactis subspecies lactis, has been found to reduce methane production by up to 40% depending on its concentration. Bacteriocins, like monensin, help to change rumen fermentation, resulting in increased propionate and reduced methane generation.

2.3 NITRATE SUPPLEMENTATION

2.3.1 NITRATE AS RUMEN MODIFIER AND AN ELECTRON ACCEPTOR FOR METHANE MITIGATION

The control of methanogenesis can be achieved by developing an alternative hydrogen sink to divert hydrogen away from methanogenesis (Joblin *et al.*,1999). To channelize the H_2 other than methanogenesis alternate hydrogen sinks are being targeted. The alternate hydrogen sinks like nitrate reduction, acetogenesis, sulphate reduction, are some of the major alternate hydrogen sinks present in the rumen. Stimulation of nitrate reduction in the rumen has very promising results and can work by competing with methanogenesis. To activate or stimulate the nitrate reduction in the rumen, nitrate has been tried as a feed supplement. The reduction of nitrate ($\Delta G=-163$ KJ mol) and sulphate ($\Delta G=152$ KJ mol) is thermodynamically favored over carbon dioxide reduction (Δ G 130 KJ/mol), which provides nitrate and sulphate-reducing bacteria a competitive advantage over methanogens as hydrogen sink (Oremland *et al.,* 1988). Feeding nitrate has dual benefits i.e., it decreases methane production and also serves as NPN (non-protein nitrogen) source for ruminants.

2.3.2 NITRATE EFFECT ON RUMEN MICROBES

Nitrate works as a rumen microbiome modifier; therefore, studies were conducted to know changes that were taking place in the rumen microbiome due to nitrate feeding. The effects of nitrate (0, 2, 4, and 6% potassium nitrate of DMI) had been shown strong inhibition on methanogens (Sophia *et al.,* 2010), and the number of methanogens reduced when nitrate was included in the diet ($P<0.001$) (Zijderveld *et al.,*2010). It was reported that *F. succinogenes* is inhibited by nitrate in the nitrate-unadapted rumen *in vitro* culture (Hulshof *et al.,*2012, Zhou *et al.,*2012). The populations of major cellulolytic bacteria *F.succinogenes, R. flavefaciens,* which contain electron transport capabilities, as well *R. albus,* which does not contain electron transport capabilities, all lowered in the rumen of goats fed nitrate and the population of methanogens also suppressed on nitrate supplementation due to toxic effects of nitrite, produced as an intermediate during the reduction of nitrate (Asanuma *et al.,* 2015).

Alaboudi and Jones (1985) observed safe acclimatization of sheep to high levels of nitrate in the diet (KNO_3 @ 2.5 g/Kg body weight per day) involved a narrowing of the ratio of nitrate and nitrite reduction activities and an increase in the proportion of nitrate-reducing rumen bacteria. Marais *et al.* (1988) found that the number of cellulolytic, xylanolytic, and total microbial populations decreased rapidly as nitrite was formed from nitrate in the digesta of the rumen. At peak nitrite values (10 mg/l), cellulolytic bacteria were reduced by 64%, xylanolytic bacteria by 25%, and total viable bacteria by 57%. They also reported that the decrease in xylanase and cellulase activities in vitro was associated with the change in the population density of microbes indicating that the nitrite is highly toxic to the fiber degrading microbes.

In the rumen, three bacterial species *Wolinella succinogenes, Veillonella parvula,* and *Selenomonas ruminantium* have been identified as specific nitrate reducers. In goats, the population size of *S. ruminantium* (13-5.6x10' cells ml) is quite high as compared to *V. parvula* (3.2x10 cells/ml) and *W. succinogenes* (1.6 x10^3 cells ml). The population density of these bacteria depends on the level of nitrate in the rumen. *W. succinogenes* and *V. parvula* are more sensitive to nitrate than *S. ruminantium*. Asanuma *et al.* (2002) using competitive PCR demonstrated a significant increase in the population density of *V. parvula and W succinogenes* by feeding nitrate (6 g/day) to goats, whereas, *S. ruminantium* remained unchanged. The type of diet also affects the population size of these nitrate reducers. Iwamoto *et al.* (2001) reported a higher rate of nitrate and nitrite reduction from the mixed ruminal microbes collected from the goats fed high roughage diet as compared to a high concentrate diet which was associated with higher nitrate and nitrite reductase activity in the former case. Asanuma *et al.* (2002) reported a higher number of W succinogenes in goats by feeding high roughage diet.

The number of *V. parvula* including *V. dispar* was greater (6.7 x10^3 vs. 3.0 x 10^3/ml) in goats fed high roughage diet than high concentrate diet, but it was not significant. On other hand, the cell numbers of *S. ruminantium* were greater (5.6x107 vs. 1.3x107/ml) in goats fed a high concentrate diet than a high roughage diet, although the difference was not statistically significant ($P<0.05$).

The amount of nitrate in the diet had a great effect on the growth of *W. succinogenes* in the rumen. The numbers of *V. parvula* and *W. succinogenes* in the rumen were higher when goats were fed high nitrate diet than a low nitrate diet (Asamuma *et al.*, 2002). The presence of nitrate favors the growth of nitrate-reducing bacteria in competition with other ruminal bacteria because these nitrate-reducing bacteria obtain energy from nitrate and or nitrite reduction and in addition, they are less sensitive to nitrite, an intermediate product of nitrate reduction (Iwamoto *et al.*, 2002).

The rumen ciliate protozoa are also sensitive to the level of nitrate or nitrite in the rumen. A numerical decrease in ciliate protozoa was observed in sheep given nitrate and in sheep inoculated with E. coli W3110 or E. coli nir-Ptac when compared with saline-infused control sheep. The inoculation of E. coli W3110 along with nitrate numerically increased the ciliate protozoa than sheep given nitrate alone (Sar *et al.,* 2005e).

The total number of rumen bacteria rose as a result of sulphate (2.6% of DM). whereas the number of methanogens was suppressed when nitrate (2.6% of DM) was fed. The number of protozoa was unaffected by the feeding of sulphate or nitrate in cross bred Texel lambs (Van Zijderveld *et al,* 2010a).

2.3.3 NITRATE EFFECT ON RUMEN FERMENTATION

In unadapted animals, dietary nitrate can suppress ruminal fermentation (Guo *et al.,* 2009), but this inhibition disappears after the animal is adapted to dietary nitrate (Zhou *et al.,*2012). The total VFAs and acetate concentration increased linearly by increasing nitrate levels in the diet of nitrate-adapted ruminants (Zhao *et al.,* 2015). Inhibition of CH_4 production by ruminal cultures has been attributed to the direct toxicity of nitrite to methanogens (Bozic *et al.,*2009; Sar *et al.,*2005).

The effects of supplementation of calcium nitrate or urea as NPN sources and Sulphur (0.8%) from sodium sulphate on rumen methane emissions showed a reduction in the methane/carbon dioxide ratio in the eructed breath of goats fed on nitrate supplemented diet as compared with animals fed on urea supplemented diet (Silivong *et al.,* 2011). Nitrate fed (25 g nitrate/kg DM) to sheep shown a 23% reduction in enteric CH_4 production per kg DM intake (Nolan et al., 2010). Feeding of nitrate (2.6% of DMI) did not affect VFA concentrations except for a decrease in branched-chain fatty acids but there was a 32 percent reduction in methane production (Zijderveld *et al.,* 2010). Supplementation of nitrate 1% and 2% of DM in rumen-fistulated steers diet did not affect ruminal ammonia nitrogen (Zhao *et al.,* 2015).

2.3.4 NITRATE EFFECT ON NUTRIENT UTILISATION

The nitrate fed to sheep at 2% of the concentrate mixture did not reduce feed intake (Pal *et al.,* 2015) and there is no change in DM intake by feeding 26 g nitrate/kg (Zijderveld *et al.,* 2010). Nitrate supplementation did not affect whole tract or ruminal DM digestibility (56.8 vs 59.4%), microbial crude protein flow (73.9 vs 58.4 g/day) when sheep fed to a diet consisted of chaffed oat hay supplemented with either 4 or 0% KNO_3 (Nolan *et al.*, 2010).

The effect of giving feed blocks containing varying quantities of urea and calcium sulphate mixture on feed intake, digestibility, and rumen fermentation in Thai native beef cattle fed rice straw was investigated by Cherdthong *et al.* (2014). Cattle receiving feed blocks containing urea calcium sulphate had the highest overall intake of DM and energy (ME, MJ/d), followed by 150, 120, and 0 g/kg DM. With the exception of acid detergent fibre, increasing quantities of urea calcium sulphate addition in feed blocks improved apparent nutritional digestibility. Silivong *et al.* (2011) compared the effect of calcium nitrate (3.8% of DM) and sulphur (0.8%) from sodium sulphate on digestibility and nitrogen balance in crossbred goats (*Bach thao* x local female) fed on a basal diet of molasses and Mimosa (*Mimosa piga*) foliage. He found that digestibility of crude protein and N retention was increased by sulphate supplementation, whereas, remained unaffected by nitrate.

Sophal *et al.* (2013) studied the effect of potassium nitrate (@5% of DM) as a source of fermentable nitrogen in local yellow cattle fed a basal diet of cassava root chips and fresh cassava foliage. They found that the DM intake was less on the nitrate diet. The apparent digestibility of DM and organic matter was reduced by 3.2 and 5.1%, respectively. while N retention per unit organic matter digested was increased by 28%. However, Sophea and Preston (2010) found no effect on live weight gain or DM feed conversion in growing goats with potassium nitrate (@0, 2, 4, 6% of DM) supplementation.

Nitrate and saponin, either alone or in combination did not reduce the digestibility of DM or NDF (Patra *et al.*, 2014) and there is no effect of nitrate feeding (2 percent of DMI) on DMI and nutrients digestibility though there was about 15 percent reduction in methane emission in buffaloes (Sakthivel *et al.*, 2011).

2.3.4 NITRATE EFFECT ON METHANOGENESIS

In cross-bred Texel lambs, Van Zijderveld et al. (2010a) investigated the influence of dietary additions of nitrate (2.6 percent of DM) and sulphate (2.6 percent of DM) on intestinal methane emissions. Over the course of four weeks, lambs were given nitrate and sulphate on a corn silage-based diet, and methane production was measured in respiration chambers. They also claimed that the decrease in methane generation was most significant immediately after nitrate feeding, but the effect was constant throughout the entire day in sulphate feeding (nitrate-32 percent, sulphate-16 percent, and nitrate + sulphate-47 percent relative to control). CH_4 production was decreased by 16% (P=0.009) in high-yielding dairy cows fed a basal diet of maize silage and replacing 15% urea with 2.2% nitrate gradually (25% per week). This reduction was not affected by time (treatment x time interaction P-0.961), indicating that the methane reducing effect was persistent over four months (Van Zijderveld *et al.*, 2010b).

Sophea *et al.* (2010) studied the effect of nitrate-N (0, 2, 4, and 6% potassium nitrate (KNO_3 of DMI) on changes in rumen gases of goat fed diet containing 40% sugar palm syrup and high rumen undegradable protein. The nitrate-N diet decreased the ratio of methane to carbon dioxide. With 6% potassium nitrate, the CH_4: CO_2 ratio was 0.0057 which was very close to the atmospheric ratio of 0.0047. Thus, the inhibitory effect of nitrate on methanogen was very strong. The results indicated that nitrate could be safely used as a rumen supplementary nitrogen source to improve animal feed intake and animal performance with another tremendous advantage of reduction of rumen methane emission. Sophea and Preston (2011) found similar findings in developing goats fed 0, 2, 4, and 6% KNO3 in place of iso-nitrogenous quantities of urea in a diet of fresh mimosa silage, rice straw, and water spinach. For 0, 2, 4, and 6 percent KNO3 supplementation, the percentage reduction in methane production was 0, 32.8, 49.2, and 60.6, respectively.

The influence of dietary nitrate on methane production was investigated in sheep given chaffed oat hay enriched with either 4 or 0% KNO3 but made iso-nitrogenous by the addition of urea (Nolan *et al.*, 2010). In the KNO3 supplemented group, methane yield (MY-L kg DMI) was reduced by 23%, and these sheep had a shorter mean fluid retention time in the rumen (MRT). MRT and MY had a strong relationship, with a shorter MRT being related with a lower MY.

Hulshof *et al.* (2010) reported that the daily methane production per animal was reduced (P<0.001) by 32% when Brazilian Bos indicus (Nelore x Guzera) steers were fed nitrate (2.2% of DM) in a total mixed ration of sugar cane and concentrates (60:40) and iso nitrogenous amounts of urea (1.2% of DM) served as control. However, DMI was not affected (P-0.14) by the addition of nitrate to the diet. On the nitrate diet, methane emission per kg of DMI was 27 percent lower (P<0.001) (13.6 vs. 18.6 g/kg DMI). On the nitrate diet (4.4 percent of GEI), methane losses were lower (P<0.001) than on the control diet (5.2 percent of GEI) (5.9 percent of GEI)

The effect of supplementation of NPN from calcium nitrate or urea, and sulphur (0.8%) from sodium sulphate on rumen methane emissions, digestibility, and nitrogen balance was studied in crossbred goats (*Bach thao* x local female) fed on a basal diet comprised of molasses and mimosa (*Mimosa piga*) foliage (Silivong, 2011) When goats were given calcium nitrate as part of a meal that included molasses and mimosa leaf, the methane carbon dioxide ratio in their eructed breath was lower than when they were given urea. Adding 0.8 percent sulphur to the diet as sodium sulphate lowered the methane carbon dioxide ratio as well, with the two supplements working in tandem.

2.4 LINSEED OIL SUPPLEMENTATION

2.4.1 LINSEED OIL AS A RUMEN MODIFIER

The inclusion of vegetable oil or lipid is one of the most promising techniques for reducing methane emission from ruminants (Beauchemin *et al.*,2008). The addition of fats or oils to ruminant diets inhibits methane generation through two methods. Protozoal inhibition, elimination of double bonds in unsaturated fatty acids, higher productivity, and increased propionate production are all part of the indirect pathway. The direct mechanism is fatty acid toxicity to methanogens. Many workers have commented on the indirect impacts of oils, which ultimately result in a reduction in methane emissions. Biohydrogenation is a hydrogen sink formed by unsaturated fatty acids, but the amount of total metabolizable hydrogen used in the biohydrogenation process of endogenous unsaturated fatty acids (0.01) is very small (0.01) when compared to that used in CO2 to methane reduction (0.48), VFA synthesis (0.33), and bacterial cell synthesis (0.12). (Czerkawski *et al.*,1986).

2.4.2 LINSEED OIL EFFECT ON DM INTAKE AND DM DEGRADABILITY

Lipids of either plant or animal origin affect degradation and digestibility of non-lipid energy sources both in the rumen and in the total digestive tract (Doreau *et al.,* 1991), but the extent of this effect varies with the nature and amount of lipids used, the type of diet, the animal species and the experimental conditions (Dong *et al.,* 1997). Ruminal degradation of structural carbohydrates can be reduced by 50% or more about 10% added fat (Jenkins *et al.*,1984). There is a significant decrease in DM and OM digestibility in dairy cows when fed with 5.7% linseed oil and extruded linseed (Martin *et al.,* 2008).

Voluntary feed intake generally decreased with a high-fat percentage in the diet (Wanapat *et al.,* 2011). Individual oil has its characteristic odor, fatty acid composition, and effect over rumen dry matter intake as well as digestibility. Dry matter intake also depends on the palatability of the offered feed, the passage rate of the feed, and the rumino-reticular motility. Oil, when reaches to rumen it forms a thin layer coating over the fibrous feed particles in the rumen (Jenkin, 2003) thus hampers the microbial attachment which results in the increased feed retention time in the rumen and decreased voluntary intake (Rumen fill) by animals, consequently daily dry matter intake. There is a decrease in DMI by 24.76% and15.66% when 5.7% of DM linseed oil and extruded linseed supplemented in silage-based diet to dairy cows (Martin *et al.*, 2008). This detrimental effect has been seen in sheep at maintenance who were fed a hay-concentrate diet supplemented with 5% (Cottyn et al., 1971) or 7% (Ikwuegbu and Sutton, 1982; Sutton et al., 1983) linseed oil. Other studies in dairy cows [3% linseed oil with either a hay-based diet (Ueda et al., 2003) or a corn silage-based diet (A. Ferlay, INRA, Saint Genès Champanelle, France, and Y. Chilliard, unpublished data)] or dry cows [2.5 percent FA from linseed or linseed oil, Doreau et al., in press)] in lambs (6.7% linseed, i.e., 2.5% FA; Machmüller *et al.,* 2000), or in sheep (10.5% linseed, i.e., 4.8% FA given 12 times/d; Wachira et al., 2000) did not show any suppression in cell wall digestibility due to lipids from linseed. Furthermore, Gonthier *et al.* (2004) found that adding 3.5 to 4 percent FA from extruded linseed to a grass and corn silage-based diet increased the total digestibility of OM and fiber. The number of additional lipids and their mode of presentation (oil vs. seed) are important determining variables for the unfavorable effect of linseed FA on digestibility, according to the results of these trials. Based on the results of a study by Ben Salem et al. (1993) in which cows were fed a meal containing 7% rapeseed oil, they claimed that the negative effect of lipids on digestion is more severe with corn silage diets than with hay diets. Around 90% of total digestible fibre in ruminants is digested in the rumen, while ruminal fibre digestion can be somewhat compensated for by digestion in the largeintestine. This is in line with prior research on other lipid sources, including linseed oil in cows (Ueda *et al.,* 2003), sheep (Ikwuegbu and Sutton, 1982), and lambs (Machmüller *et al.,* 2000).

2.4.3 EFFECT OF LINSEED OIL ON NUTRIENT DIGESTIBILITY AND VFA PRODUCTION

Martin *et al.* (2008) investigated the effects of three different types of linseed fatty acids (FA) on total tract digestibility and dairy cow performance. Eight multiparous nursing Holstein cows were randomly assigned to one of four diets: control (C), crude linseed (CLS), extruded linseed (ELS), or linseed oil (LSO) at the same FA level, or the same diet with crude linseed (CLS), extruded linseed (ELS), or linseed oil (LSO) (5.7 percent of dietary DM). Each study lasted four weeks. The supplemented diets had significantly worse whole tract NDF digestibility ($P<0.001$) than the control diets (-6.8% on average; $P<0.001$). CLS had no effect on DMI ($P>0.05$), whereas ELS and LSO (-3.1 and 5.1, respectively) did. CLS had no effect on DMI ($P>0.05$), while ELS and LSO did (-3.1 and 5.1 kg/d, respectively; $P<0.001$).

Benchaar *et al.* (2012) observed the effect of linseed oil (LO) supplementation on nutrient digestibility, forage (i.e., timothy hay) *in Sacco* ruminal degradation, ruminal fermentation characteristics, protozoal populations in dairy Cows. Four ruminally cannulated, lactating cows were fed a total mixed ration (50:50 forage: concentrate) without supplementation (control, CTL), or supplemented with LO at 2, 3, or 4%. Supplementation with LO did not affect DM intake and apparent total-tract digestibility of nutrients. Dietary LO supplementation had no effect on ruminal pH, ammonia, or total volatile FA concentrations. The amount of change in the volatile FA pattern and effective ruminal degradability of timothy hay DM was minimal. Increasing the amount of LO in the diet had no effect on the total number of protozoa or the distribution of taxa. Guyader *et al.* (2015) studied the effect of linseed oil and nitrate fed alone or in combination on diet digestibility in cows. On a DM basis, the diets were administered as follows: 1) control (CON 50 percent natural grassland hay and 50 percent concentrate), 2) CON with 4% linseed oil (LIN), 3) CON with 3% calcium nitrate (NIT), and 4) CON with 4% linseed oil with 3 percent calcium nitrate (LIN+NIT). Linseed oil and nitrate reduced ($P<0.01$) CH_4 emissions (g/kg DMI) by 17 and 22 percent, respectively, when fed alone, and by 32 percent when fed together, when compared to the CON diet. In comparison to the CON diet, the LIN diet raised ($P = 0.02$) propionate percentage and decreased ($P = 0.03$) ruminal protozoa concentration. Except for linseed oil, which tended to diminish (P 0.10) fibre digestibility, dietshad no effect (P >0.05) on total tract digestibility of nutrients. Nitrogen balance (% of N intake)was favourable in all diets, but linseed oil retention was lower ($P<0.03$).

2.4.4 LINSEED OIL EFFECT ON RUMEN FERMENTATION PARAMETERS

Rumen protozoa are also involved in methanogenesis (Kreuzer *et al.*,1986) and are responsible for 9 to 25% of methanogenesis in rumen fluid probably because of their association with ecto and endosymbiotic methanogenic bacteria. Methane release was greatly reduced by coconut oil, sunflower seed, and linseed, as was the amount of ciliate protozoa, which are known to provide a habitat for rumen methanogens (Finlay *et al.*, 1994). C_{18} unsaturated acid (provided as linseed oil) has a better antiprotozoal impact than saturated C_{12} to C_{14} acids (coconut oil) (Newbold *et al.*, 1988). For dairy cows, the efficiency of microbial protein synthesis in the rumen rose linearly with the degree of unsaturation in dietary fat, but ruminal protozoal populations decreased linearly with increasing the degree of unsaturation in dietary lipids (Pantoja *et al.*, 1994). (Oldick *et al.*, 2000).

During the fattening period, Eugene *et al.* (2011) conducted a study to measure CH_4 generation from bulls fed a feedlot diet rich in either fiber (F) or starch and lipid (SL). Fifty-six Charolais bulls (259±9.4 days old and 339 ±8.2 kg LW) were fattened for up to 18 months after being randomly assigned to one of two diets and blocked with four replicate pens/diet based on LW and age. Barley straw was used in both treatments, along with a concentrated mixture rich in fiber, starch, and fat. The pH of the rumen did not alter between bulls given SL and bulls fed F. In bulls fed SL, the ruminal molar concentration of total VFA was lower ($P<0.001$) than in bulls fed F. Bulls fed SL exhibited reduced ruminal acetate proportions ($P<0.0001$), with the magnitude of the difference being greater at the start of fattening than at later periods ($P<0.001$). Bulls given SL showed higher proportions of propionate ($P<0.05$) at the start and midway of fattening, as well as higher butyrate <(P0.05) at the start than bulls fed F. The acetate to propionate ratio was lower in SL-fed bulls than in F-fed bulls, and the magnitude of the difference was greater at the start of fattening than at other sample points ($P<0.05$).

Veneman *et al.* (2015) studied the effects of dietary LO (linseed oil) and NO_3 supplementation on methane generation and rumen fermentation in lactating dairy cows in two separate studies with varied geographical locations and basal diets. In both cases, the treatments were a control diet and a diet supplemented with LO at 4% of feed DM and NO_3 at 2% DM from Bolifor® (Yara, Finland). In both experiments, urea is used to keep the diets isonitrogenous and a rumen inert fat source to keep the fat content equal. Methane emissions in g per day (both 21 percent; $P<0.02$) and per kg DMI (16 and 13 percent; $P<0.04$ in exp1 and exp2, respectively) were significantly reduced when NO_3 was fed. Only in exp1, NO_3 feeding considerably reduced methane emissions per kg FPCM. Furthermore, feeding NO_3 enhanced hydrogen emissions significantly in both studies ($P<0.03$), but overall hydrogen emissions were minimal (2.9 and 3.2g/day for Exp1 and Exp2, respectively), which could not explain why methane reductions were below the stoichiometric potential of nitrate to reduce methane (68 and 66 percent for Exp1 and Exp2 respectively). Total VFA concentrations in the rumen were unaffected by LO or NO_3, but NO_3 increased the acetate to propionate ratio in both trials ($P<0.02$). When fed NO_3, these findings show that the distribution of metabolic hydrogen changes between methanogenesis, nitrate reduction, and the generation of particular VFAs. In both experiments, NO_3 reduced methane emissions by a similar amount (21 percent each day), whereas LO did not affect emissions. When LO was added to diets, it did not affect ruminal liquor pH or total VFA concentrations ($P > 0.10$). When they introduced LO at 3% to dairy cow forage- and concentrate-based diets, Ueda et al. (2003) found no effect of LO supplementation on ruminal liquor pH or total VFA content. When Shingfield *et al.* (2011) added 3% LO to a corn silage-based diet for developing steers, they got similar outcomes. Broudiscou *et al.* (1994) showed a decrease in total VFA concentration in sheep fed a forage-based diet supplemented with 6% of LO (55:45 F: C).

Supplementation of unprotected and highly unsaturated fats to ruminant diets is thought to lower the acetate proportion and acetate: propionate ratio in the rumen, with an antimicrobial impact of PUFA-rich oils being one possible cause (Jenkins and Jenny, 1992). Previous studies reported a shift in VFA patterns toward proportionally higher propionate and less acetate when sheep (Broudiscou *et al.*, 1994) and dairy cows (Ueda *et al.*, 2003) were supplemented with LO. However, other studies reported no change in VFA patterns when dairy cows were fed LO-supplemented diets (Doreau et al., 2009; Shingfield *et al.*, 2011). The source of fodder supplied may explain the disparity across research for the effects of LO on VFA patterns once again. Indeed, when LO was supplemented to a hay-based diet (Ueda *et al.*, 2003), the effect on VFA patterns was not the same as when it was given to a corn silage-based diet (despite a similar level of supplementation (i.e., 3 percent, DM basis) (Doreau *et al.*, 2009). This is supported by the findings of Ben Salem *et al.* (1993), who found that when rapeseed oil was introduced to a hay-based diet, the proportions of acetate and propionate remained unchanged, however when corn silage was used as the primary forage source, the proportions of these VFA changed. LO supplementation did not affect ruminal ammonia concentrations ($P > 0.05$). (Benchaar *et al.*, 2012). Previous experiments with LO have yielded a mixed bag of findings. LO supplementation did not influence ammonia concentration according to Doreau *et al.* (2009), however, Ueda et al. (2003) found that ruminal ammonia concentration was higher in LO-supplemented dairy cows than in control cows. In other investigations, sheep treated with various amounts of LO showed a drop in ammonia levels (Ikwuegbu and Sutton, 1982; Broudiscou *et al.*, 1994).

2.4.5 LINSEED OIL EFFECT ON METHANE PRODUCTION

Using the SF6 tracer approach, Martin *et al.* (2008) investigated the effect of three kinds of linseed fatty acid (FA) administration on methane emission in dairy cows. Eight multiparous lactating Holstein cows were randomly assigned to one of four dietary treatments: the control diet (C), which consisted of corn silage (59%), grass hay (6%), and concentrate (35%); the same diet with crude linseed (CLS), extruded linseed (ELS), or linseed oil (LSO) at the same FA level; and the same diet with crude linseed (CLS), extrude (5.7 percent of dietary DM). Each experiment lasted four weeks. When compared to C, all types of linseed FA significantly reduced daily CH4 emissions (P 0.001), albeit to varying degrees (-12 percent with CLS, -38 percent with ELS, -64 percent with LSO). Linseed FA appears to be a viable dietary option for inhibiting ruminal methanogenesis. The way linseed FA is presented (oil vs seeds) has a big impact on how much methane is produced by dairy cows.

Benchaar *et al.* (2015) examined the effect of linseed oil (LO)supplementation to red clover silage (RCS)- or corn silage (CS)-based diets on enteric CH_4 emissions. Twelve rumen-cannulated lactating cows were fed RCS. or CS-based diets with 60:40 roughage to concentrate ratio and supplemented with or without linseed oil (4% of DM). Linseed supplementation to the RCS-based diet reduced enteric CH_4 generation (9%) and CH4, energy losses (11%) while having no negative effect on performance. The addition of linseed oil to the CS-based diet decreased methane production to a larger extent (26%) and CH_4, energy losses (23%).

Guyader *et al.* (2015) studied the effect of linseed oil and nitrate fed alone or in combination on methane (CH_4) emissions in cows. The experiment was conducted using four multiparous nonlactating Holstein cows. The diets were given on a DM basis 1) control (CON; 50% concentrate and 50% natural grassland hay), 2) CON with 4% linseed oil (LIN), 3) CON with 3% calcium nitrate (NIT), and 4) CON with 4% linseed oil plus 3% calcium nitrate (LIN+NIT). Linseed oil and nitrate reduced ($P< 0.01$) CH_4 emissions (g/kg DMI) by 17 and 22 percent, respectively, when fed alone, and by 32 percent when fed together, when compared to the CON diet. Throughout the day, the LIN diet lowered CH_4 generation. The NIT diet significantly reduced CH_4 generation 3 hours after feeding, while simultaneously increasing rumen dissolved H_2, indicating that nitrate is more than an electron acceptor. The combination of linseed plus nitrate also increased H_2 concentrations in the rumen. They demonstrated an additive effect between nitrate and linseed oil for reducing methanogenesis in cows without altering diet digestibility.

A decrease in the hydrogen supply to methanogens, a shift in fermentation toward propionate at the expense of acetate, and/or a drop in protozoa that create hydrogen can all help to reduce rumen methanogenesis (Morgavi *et al.,* 2010).

The fact that linseed oil reduces CH_4 emissions in dairy cows backs up in vitro findings (Broudiscou and Lassalas, 1991). Growing lambs supplemented with 6.7 percent crushed whole linseed (i.e., 2.5 percent oil; Machmüller *et al.,* 2000) or sheep at maintenance receiving 5 percent linseed oil in intraruminal continuous infusion showed a depressive effect of linseed FA on in vivo CH_4 emissions, as measured in respiratory chambers (Czerkawski *et al.,* 1966a).When the same amount of linseed oil FA is delivered continually rather than once, the deleterious effect of FA on methanogenesis is reduced (Czerkawski *et al.,* 1966b). PUFA in free oil interacts with microbes in the rumen more quickly than FA in seeds. This is illustrated by the fact that the VFA pattern in oils shifts more toward propionate than in seeds (Jouany et al., 2000). The way of dispensing of the oil utilised in this trial for the treatment diet may have highlighted this effect. Thus, a shift in fiber digestion from the rumen to the large intestine may have occurred for the linseed oil in the diet, and, as a consequence, less methane was produced. The SF6 technique's removal of hindgut methane likely resulted in an underestimating of methane production for linseed oil in the diet when compared to the other diets. When comparing diets, we can assume that differences in fibre processed in the rumen are bigger than differences in the entire tract. This was confirmed by Sutton et al. (1983), who found that lambs treated with 7% linseed oil had a greater drop in OM digestion in the rumen (19 points) than in the whole tract (3 points). Polyunsaturated fatty acids reduce methane synthesis by causing toxicity in microorganisms involved in fiber digestion and hydrogen production, such as protozoa and cellulolytic bacteria (Doreau and Ferlay, 1995). (Nagaraja *et al.,* 1997). This impact, which is seen with all long-chain FA, is most likely due to an action on the cell membrane, especially in gram-positive bacteria. Linolenic acid has been demonstrated to be highly harmful to the three cellulolytic bacterial species (Fibrobacter succinogenes, Ruminococcus albus, and Ruminococcus flavefaciens) in vitro due to its disruption of cell integrity (Maia *et al.,* 2006). Furthermore, as demonstrated in vitro using linseed oil hydrolysate, PUFA may have a direct harmful effect on methanogens that require hydrogen for methane synthesis (Prins *et al.,* 1972). In another investigation, free hydrogen accumulated in the gas mixture, inhibiting cellulolytic bacteria development (Wolin *et al.,* 1997). Long-term adaptation of the rumen microbiota to oil supplementation may be conceivable, and the long-term persistence of methane suppressing diet modifications has been identified as a major concern (Woodward *et al.,* 2006; Grainger *et al.,* 2008).

2.5 ANTHRAQUINONE ($C_{14}H_8O_2$) SUPPLEMENTATION

2.5.1 ANTHRAQUINONE ($C_{14}H_8O_2$) AS A RUMEN MODIFIER

Anthraquinone (9, 10- dioxo anthracene) is a naturally occurring aromatic organic compound found within some plants. Anthraquinone (AQ) is a derivative of anthracene and has the appearance of yellow or light grey solid crystalline powder. There are different natural sources of AQ such as Cascara sagrada bark (Rhamnus purshiana), *Cassia fistula* (Golden shower), *Artemisia scoparia* (Saffron), *Kniphofia foliosa* roots, and *Aloe succotrina* (Burn plant). Anthraquinone is the main active component of aloe (Aloe succtrina) and senna (*Senna Alexandria*) oils (Ebrahimi *et al.,*2011).

AQ derivatives are used as laxatives, besides they also have other medicinal properties such as antibacterial, antiviral, diuretics, and antiparasitic (Godding, 1976). They can be divided into two groups: soluble and insoluble in water. The non-ionic compounds are largely insoluble in aqueous systems, while ionic derivatives, such as di-alkali metal salts, are largely soluble in water (Ballinger, 2004). AQ has the property to directly inhibit methanogens (Garcia et al, 1996) and thereby reduces methane production. The great anti-methanogenic property of rhizomes and roots of *Rheum offianale* (rhubarb) was attributed to the presence of AQ in this medicinal plant (García-González *et al.,* 2008a, b).

2.5.2 ANTHRAQUINONE EFFECT ON METHANE AND HYDROGEN PRODUCTION

Ruminant methanogens use the methanogenesis pathway (Hydrogenotrophic) to convert H_2 and CO_2 (generated by bacteria, protozoa, and anaerobic fungus) into CH_4 (Patra *et al.,*2010). For the synthesis of methane from H_2 and CO_2, methanogenic archaea require the methyl coenzyme M reductase (MCR). In a variety of situations, the MCR is utilised as a reliable marker of methanogenesis (Luton *et al.,*2002; Palacio *et al.,*2013).

The Rhubarb compounds 9,10-Anthracenedione, 1,8-dihydroxy-3-methyl (6.692 kcal/mol), phthalic acid isobutyl octadecyl ester (5.26), and di iso-octyl phthalate (5.61) have more specificity towards the methyl-coenzyme M reductase binding site and could be a potent anti-methanogen inhibitor, according to Among the chemicals produced from the Rhubarb, this study suggests that three compounds have the potential to develop an anti-methanogenic drug (Arokiyaraj *et al.*,2019). 9,10 anthraquinone (AQ) has been proven to partially inhibit methane *in vitro* experiments (Garcia-Lopez et al.,1996). Increasing levels of AQ caused a linear and quadratic decrease in the total gas and methane production and an increase in hydrogen concentration (Ebrahimi *et al.*,2011).

2.5.3 ANTHRAQUINONE EFFECT ON VFA PRODUCTION IN THE RUMEN

Anthraquinone when supplemented, there was no increase in propionate production, although there was an increase in the A/P ratio (Sirohi et al.,2010). Total VFA and acetate concentrations were unaffected by the addition of 4 ppm AQ, whereas propionate and valerate concentrations were dramatically raised. Total VFA, propionate, valerate, acetate, and iso-butyrate concentrations were all decreased at the maximum dose of AQ (8 ppm). Because the concentration of propionate was increased without a substantial change in acetate concentration, the acetate-to-propionate ratio was lowest at the AQ level of 4 ppm.and did not affect pH and ammonia nitrogen under *in vitro* as well as *in vivo* conditions (Ebrahimi *et al.*,2011).

CHAPTER- 3

MATERIALS AND METHODS

MATERIALS AND METHODS

The materials and experimental techniques employed to estimate the **“Comparative Evaluation of Combination of Methanogenic Inhibitors on Enteric Methane Emission and Nutrient Digestibility in Sahiwal and Gir Calves”** are presented in this chapter.

3.1. In vivo study

3.1.1. Location of experiment

The experiment was conducted at the Livestock research center of ICAR-National Dairy Research Institute, Karnal, Haryana located at 29042′20″ sec N and 76058′52.5 ″ sec E at an altitude. Minimum and maximum ambient temperature range from a near freezing point in winter to 42^0C in summer with a diurnal variation in the order of 15-20^0C along with an annual rainfall of 700 mm. This study was conducted from November-2020 to April-2021 with daily minimum and maximum temperatures averaging 4^0C and 36^0C, respectively. The experimental protocol including handling and management of animals complied with the prior approval of the Institutional Animal Ethics Committee.

3.1.2 Selection and distribution of experimental animals

Total 12 healthy Sahiwal calves (6) and Gir calves (6) of 6-12 months age were selected from the herd of LRC, Karnal and divided into 4 groups of 3 animals each, based on their average body weight, (Table 3.1).

✓ Control

✓ Treatment

Switch over design (Period I and Period II) was followed.

Period I

Sahiwal calves

N=6

Sahiwal Control (n=3)

control diet as per ICAR 2013

Sahiwal Treatment (n=3)

control diet +potassium nitrate 1% of total DMI, linseed oil 0.5% of Concentrate mixture and anthraquinone (4ppm)

Gir calves

N=6

Gir Control (n=3)

control diet as per ICAR 2013

Gir Treatment (n=3)

control diet +potassium nitrate 1% of total DMI, linseed oil 0.5% of Concentrate mixture and anthraquinone (4ppm)

Period II

Animals were switched over as mentioned earlier after the trial and collection of samples.

3.1.3 Housing and management of experimental animals

All animals were tethered individually using cotton rope and housed in a well-ventilated shed with an arrangement for individual feeding. During an adaptation period of 14 days, all animals were dewormed by drenching anthelmintic solution containing fenbendazole (panacur®, Intervet, India) @ 10 mg/kg BW and provided with a control diet to ascertain their dry matter intake. Healthy surroundings and proper sanitary conditions with 24-hour observation were maintained throughout the experiment by regular cleaning of the floor and mangers.

Table 3.1 Details of experimental animals

Control/treatment	Animal no.	Initial bodyweight (kg)	Age in months
Sahiwal			
Control	2809	90.9	10.8

	2804	94.2	11.9
	2808	114.6	11.4
Average		**99.90±5.24**	**11.38±0.22**
Treatment	2807	128.2	11.6
	2803	128.4	12.7
	2805	145.7	11.9
Average		**134.10±4.10**	**12.06±0.24**
Gir			
Control	99	93	9.6
	101	114.5	9.2
	103	120	8.0
Average		**109.17±5.82**	**8.96±0.33**
Treatment	98	136	9.6
	96	175.2	11.3
	102	177.6	8.9
Average		**162±9.53**	**9.94±0.50**

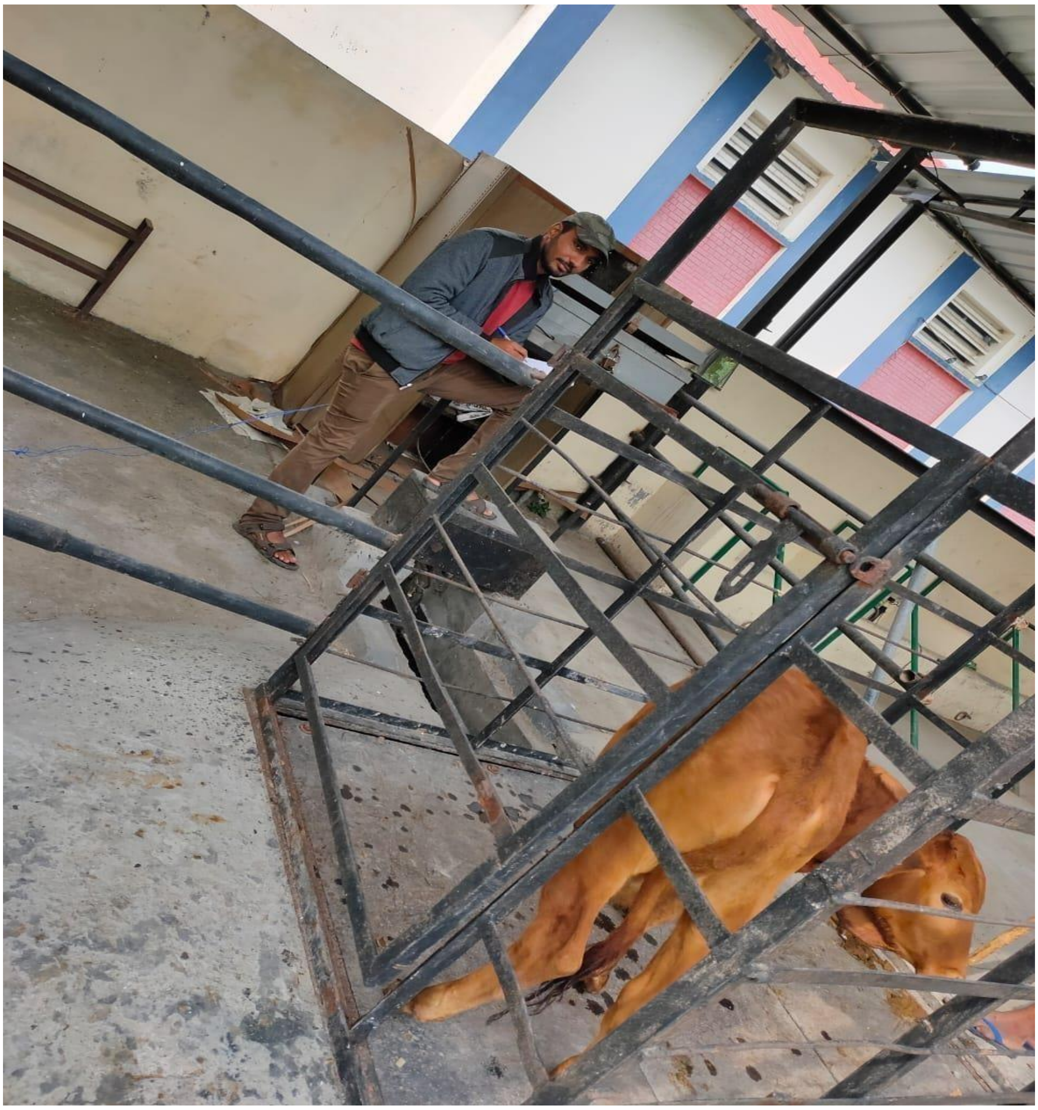

Plate 3.1 Bodyweight measurement of experiment animals

3.1.4 Feeding of experimental animals

All the animals were fed as per their nutrient requirements as per ICAR (2013) feeding standards. Animals in all the groups were fed on chopped oat particle size: (2.0-2.5 cm), threshed wheat straw (particle size: 1.5 -2.0 cm) and concentrate mixture. Green Oat forage was supplied by the farm section of the institute and was chopped freshly to feed experimental animals once daily at 11.00 am. The total daily concentrate mixture was divided into two portions and fed at 9.30 am and 4 pm. Wheat straw was offered once daily at 9.30 am. All animals were provided with fresh tap water for drinking ad libitum at 6.00 am 12.00 pm and 4.00 pm.

3.2 Parameters studied during period 1 and period 2

3.2.1. Sample collection and preparation

Feeds and fodder samples were collected weekly during the experiment and dried in a hot air oven at 70^0 C for 24 hrs till a constant weight was attained. The dried samples were groundin an electric mixer grinder. The ground samples were stored in a Ziplock bag, labeled properly,and kept for further analysis.

3.2.2. Proximate analysis (AOAC, 2005)

3.2.2.1. Dry matter (DM)

Apparatus:

Aluminum moisture cup, hot air oven, desiccator, electronic balance, tongs, spatula.

Procedure:

A known quantity of representative sample of feed ingredient was weighed in a pre-weighed moisture cup and the cup was placed in a hot air oven at 100 ± 5^0C. Weight of dried sample after drying was estimated and the dry matter was calculated as follows:

$$\mathrm{DM}(\%) = \frac{a}{b} \times 100$$

Where,

a = weight of the sample after oven drying

b = fresh weight of the sample

3.2.2.2 Total ash (TA)

Apparatus:

Silica crucible, hot plate, muffles furnace, electronic weighing balance, and tongs.

Procedure:

A known quantity of sample (about 2.5-5 g) was taken in a pre-weighed silica crucible. After charring the sample on the heater (till the smoke disappeared), the crucible was kept in a muffle furnace for ignition at 550°C for 2-3 h. The crucible was removed after cooling off a furnace, kept in a desiccator, and weighed again to find out the weight of the ash. The ash content was calculated as given below:

$$\text{TA}\ (\%) = \frac{(\text{Wt. of crucible} + \text{ash after cooling}) - \text{Wt. of crucible}) \times 100}{\text{Wt. of the sample (g)}}$$

3.2.2.3 Organic matter (OM)

Procedure:

OM was determined by subtracting the total ash content from 100.

$$\text{OM}\ (\%) = 100 - \text{TA}\ (\%)$$

3.2.2.4 Crude protein (CP)

Apparatus:

Digestion tubes, digestion unit, Kjeldahl distillation apparatus, Erlenmeyer flasks, titration assembly, burette.

Reagents:

Digestion mixture (Na_2SO_4 and $CuSO_4$ in the ratio of 9:1), 40% NaOH solution (400 g NaOH pellets dissolved in distilled water and volume made to 1000 ml), concentrated H_2SO_4 (98% purity and specific gravity 1.84)), 2% boric acid indicator solution (20 g boric acid dissolved to 1 L and added with 10 ml 0.2% bromocresol green and 20 ml 0.1% methyl red indicators), N/100 H_2SO_4 solution.

Procedure:

Total nitrogen was measured by the micro Kjeldahl method. A known quantity of sample (about 0.5-1 g) was taken in digestion tubes and digested with 20-30 ml concentrated H_2SO_4 and 2-3 g of digestion mixture till the solution became colorless. After digestion, the contents were cooled and volume was made to 100 ml. An aliquot (10 ml) was distilled in the Kjeldahl distillation apparatus (KELPLUS Nitrogen Analyzer) after adding 10-15 ml of 40% NaOH solution. About 60-75 ml of distillate (light green color) was collected into an Erlenmeyer flask containing 10 ml of 2% boric acid with indicator solution. The distillate was then titrated against standard N/100 H_2SO_4 solution and the endpoint was recorded when the color changed to slight pink. The volume of N/100 H_2SO_4 solution used in titration was recorded.

Calculation:

$$\mathrm{N}\ (\%) = \frac{0.014 \times \mathrm{N} \times \mathrm{V} \times \mathrm{D}}{\mathrm{A} \times \mathrm{W}} \times 100$$

Where, N - Normality of H_2SO_4

V - Volume of H_2SO_4 used

D - Volume made (ml)

A - Aliquot took (ml)

W - Wt. of the sample (g)

The crude protein (%) of the sample was calculated by multiplying the N content with the factor 6.25. This was based on the principle that all the proteins contain 16% nitrogen.

3.2.2.5 Ether extract (EE)

Apparatus:

Soxhlet's extraction apparatus, oil flask, thimble, hot air oven, desiccator, weighing balance.

Reagent:

Petroleum ether (boiling point - 40-60°C)

Procedure:

A known quantity of ground sample (about 3 g) was taken in a cellulose thimble and extracted for 8 hours with petroleum ether in Soxhlet's extraction apparatus attached to a pre-weighed oil flask. The oil flask was removed and after evaporating the excess of ether, it was dried overnight in a hot air oven (100±5°C). The flask was cooled in a desiccator and weighed to a constant weight. The difference between the two weights gave the amount of ether extract in the sample.

Calculation:

$$\text{EE (\%)} = \frac{(\text{Wt.of oil flask with ether extract} - \text{Wt.of empty oil flask}) \times 100}{\text{Wt.of sample}}$$

3.2.2.6 Crude Fiber

Samples after ether extraction were transferred to a spoutless beaker of 1-liter capacity. Two hundred ml 1.25 percent H_2SO_4 was added and refluxed on a hot plate for 30 minutes. The sample was filtered through a double layer of muslin cloth and again transferred to the same beaker and refluxed again with 1.25 percent NaOH solution for 30 min. The residue was washed thoroughly with hot water. The residue left after alkali and acid treatment was transferred quantitatively to a China crucible and dried, weighed, and ignited in a muffle furnace at 600°C for 1 h. Loss in weight after the ignition was calculated as CF and expressed on a percentage basis.

3.2.2.7 Estimation of cell wall constituents

The cell wall constituents of feed and feces (During the period I and II) were determined according to methods suggested by Van Soest *et al.* (1991). Neutral detergent fiber (NDF) was determined with decalin and sodium sulfite while acid detergent fiber (ADF) was analyzed with decalin. Hemicellulose was calculated as the difference between percent NDF and ADF on DM basis.

3.2.2.7.1 Neutral detergent fiber (NDF)

Preparation:

Composition of neutral detergent solution

Sodium lauryl sulphate USP :30g

Disodium EDTA dehydrate crystals :18.61g

Sodium borate decahydrate :6.81g

Disodium hydrogen phosphate, anhydrous :4.56g

2-ethoxy ethanol :10 ml

Distilled water :1000ml

EDTA and sodium borate were taken in a large beaker with 100-200 ml hot distilled water and dissolved by heating along with stirring by a glass rod. Sodium lauryl sulphate and 2-ethoxy ethanol were added to obtain a clear solution. In a separate beaker, disodium hydrogen phosphate was dissolved with 100 ml hot distilled water by gentle heating. Both the solutions were mixed properly by constant stirring; volume was made up of one liter and checked for pH (6.9-7.1).

Procedure:

A weighed quantity (About 0.5-1.0 g) of ground sample was taken in a spout-less beaker. About 100 ml of neutral detergent solution was added to it and refluxed for one hour after the initiation of boiling. Contents were filtered through a pre-weighed sintered glass crucible (Grade-1) and washed repeatedly with hot water followed by 2 washings with acetone. The sintered crucible containing residue was then dried overnight in a hot air oven (100±1°C) and weighed after cooling in a desiccator. The difference in the weight of crucible plus residue and that of the empty crucible was recorded as NDF and expressed on a DM basis.

$$\text{NDF}\ (\%) = \frac{(\text{Weight of crucible} + \text{NDF}) - \text{Weight of crucible}}{\text{Weight of sample (DM basis)}} \times 100$$

3.2.2.7.2 Acid detergent fiber (ADF)

Composition of acid detergent solution

Cetyl trimethyl ammonium bromide	20 g
1 N sulphuric acid	1000 ml

Procedure:

A weighed quantity (about 1.0 g) of dried ground sample was taken in a 600 ml spoutless beaker and 100 ml of acid detergent solution was added to it. Beaker contents were refluxed for one hour after the onset of boiling. The contents of the beaker were then filtered through a pre-weighed sintered glass crucible and washed several times with hot water followed by two washings with acetone. The crucible was dried in a hot air oven at 100±1°C overnight and weighed after cooling it in a desiccator to know the amount of ADF (%) on a DM basis

$$(\%) = \frac{(\text{Weight of crucible} + \text{ADF}) - \text{Weight of crucible ADF}}{\text{Weight of sample (DM basis)}} \times 100$$

3.2.2.8 Hemicellulose

Hemicellulose was soluble in ADS and was calculated by subtraction of ADF from NDF as follows:

Hemicellulose (%) = NDF (%) – ADF (%)

3.3. Animal related parameters

3.3.1. A. Fortnightly body weights

Fortnightly all animals were weighed on an electric scale, before feeding and watering in the morning on two consecutive days at the beginning of experimental feeding and thereafter at fortnightly intervals during the experimental feeding of 140 days and before and after each digestibility trial.

3.3.2. B. Digestibility trial

To assess the nutrient digestibility and plane of nutrition, a Digestibility trial of five days collection period was conducted twice during the last month of period 1 and period 2 of the feeding trial. The trial was conducted by housing all the animals in cages individually with arrangements for the quantitative collection of faeces. Animals were given two days of adaptation in the cages before commencing the actual collection.

3.3.2.B.1. Collection, processing, and storage of faeces feeds and refusal samples

Representative samples of feed offered, and residue left and faeces were collected in the morning and an aliquot was kept for drying and another for nitrogen estimation. The dried material obtained was pooled animal-wise and ground to pass through a 1 mm sieve and stored in Ziplock bags and preserved for proximate analysis. The total amount of faeces voided by each animal during 24 h was recorded. An aliquot (1/50 in duplicate) was dried daily in clean aluminum trays at 65^0C for 48 h for DM estimation. For N estimation, a fecal aliquot (1/500) was stored in wide-mouthed polypropylene bottles containing 25 ml of 25% H_2SO_4. After completion of the digestibility trial, the weight of the bottles was taken and feces with acid werecalculated by subtracting the empty weight of the bottle from the final weight. A suitable quantityof dung (1.5 g) from properly mixed and the pooled sample was subjected for N estimation by the Kjeldahl method.

3.3.2. B.2 Analytical procedures for digestibility trial

The pooled and dried samples of feed offered, residue, and faeces were analyzed for chemical composition like DM, OM, CP, and EE (AOAC, 2005) and cell wall fractions like NDF and ADF (Van Soest et al., 1991) detailed in -------------- --.

3.3.2. B.3 Calculation of nutrient digestibility

The apparent digestibility of various nutrients was calculated as follows:

$$\text{Digestibility (\%)} = \frac{(\text{Nutrient intake} - \text{Faecal excretion}) \text{ X100}}{(\text{Nutrient intake})}$$

3.3.2. B.4 Calculation of protein value of diet

Digestible crude protein (DCP) was used to indicate the protein value of the diet, as follows:

$$\text{DCP (\%)} = \text{CP content in the ration (\%)} \times \text{CP digestibility (\%)}$$

3.3.2. B.5 Calculation of energy value of diet

Total digestible nutrient (TDN) was used to calculate the energy value of the diet as follows:

TDN (%) = [Digestible CP intake (kg) + digestible EE intake (kg) × 2.25 + digestible CF (kg) + digestible NFE (kg)×100/DM intake

3.4 Rumen Fermentation Patterns in an *In Vivo* study

a. Feeding and watering of animals

Water was offered 3 h before the first withdrawal of rumen liquor and feeding was done after collection of sample.

b. Sampling of rumen liquor

Rumen fluid samples were collected from different parts of the rumen of each animal at 3h post-feeding, mixed thoroughly, filtered through four layers of muslin cloth, and pH of strained rumen liquor (SRL) was recorded immediately. NH_3- N and total N_2 in the collected samples were measured on the day of collection. Samples were acidified with few drops of 25% H_2SO4 and refrigerated till estimation of TVFA and IVFA.

3.4.1 Methods of analysis

3.4.1.1 pH of strained rumen liquor

The pH of SRL was recorded using a digital pH meter.

3.4.1.2 Total volatile fatty acid (TVFA) concentration

TVFA concentration in SRL was measured according to the method of Bamett and Reid (1957). Two ml of SRL sample was taken in Markham distillation apparatus and 2 ml of oxalate buffer (equal amount of 10 % potassium oxalate and 5 % oxalic acid solution) was added. About 100 ml distillate was collected in a conical flask kept in an ice bath. The distillate was titrated against 0.01 N NaOH by adding few drops of phenolphthalein indicator and TVFA wascalculated as follows:

$$\text{TVFA (mmol/100 ml SRL)}=\frac{\text{The volume of 0.01N NaOH used}}{\text{The volume of the sample taken}}\times 100$$

3.4.1.3 Individual volatile fatty acids (IVFA)

The supernatant (4 ml) was treated with 25% meta-phosphoric acid (1 ml) and kept overnight at 4°C (Erwin et al., 1961). Thereafter, it was centrifuged at 3000 rpm for 10 min and used for the IVFA estimation using a gas chromatograph (Nucon 5700, India) equipped with a flame ionization detector (FID) and stainless-steel column (length 4: od 1/4"; i.d 3 mm) packed with Chromosorb 101. The temperature of the injection port, column, and detector was set at 200, 180, and 210°C. respectively. The flow rate of carrier gas (N_2) through the column was 40 ml/min; and the flow rate of H_2 and air through FID was 30 and 300 ml/min, respectively. Sample (3 ul) was injected through the injection port using a Hamilton syringe (10 ul). Different VFA's were identified based on their retention time area covered on the monitor and their concentration (mM/L) was calculated by comparing the retention time as well as the peak area of standards after deducting the corresponding blank values. Standard VFAs solution prepared as 60, 30, and 10 mM/100 ml acetic, propionic and, butyric acid respectively. Stoichiometrically CH_4 production was also calculated based on IVFA produced (Wolin, 1960)

$$\text{Acetate (mM/L)}=\frac{\text{Area of sample}\times 60\times 10}{\text{Area of standard}}$$

$$\text{Propionate (mM/L)}=\frac{\text{Area of sample}\times 30\times 10}{\text{Area of standard}}$$

$$\text{Butyrate (mM/L)}=\frac{\text{Area of sample}\times 10\times 10}{\text{Area of standard}}$$

3.4.1.4 Estimation of Ammonia N (NH_3-N)

Five mL of SRL was taken in the distillation assembly and 5 mL of 40% NaOH. The distillate was collected in a conical flask containing 10mL of 2% boric acid solution having a mixed indicator and titrated against 0.01 N H_2So_4. NH_3-N was calculated as follows;

$$\mathrm{NH_3\text{-}N\ (mg/100ml\ SRL)} = \frac{Vol.of\ acid \times normality\ of\ acid \times\ Dilution\ factor \times\ 0.014}{\text{Volume of SRL}} \times 100 \times 1000$$

3.4.1.5 Total nitrogen (Total-N) concentration

Two ml of SRL was taken in Kjeldahl digestion flask, digested by adding 10 ml concentrated sulphuric acid along with digestion mixture. Digested material was diluted with distilled water up to 100 ml in a volumetric flask. An aliquot (10 ml) of the digested sample was taken in distillation apparatus along with 15 ml 40 % NaOH. The distillate was collected in a conical flask containing 20 ml of 2 % boric acid solution having mixed indicator, which was titrated against 0.01 N H_2SO4. Total-N was calculated as follows:

$$\mathrm{Total\text{-}N\ (mg/100ml\ SRL)} = \frac{Vol.of\ acid \times normality\ of\ acid \times\ Dilution\ factor \times\ 0.014}{\text{Volume of SRL}} \times 100 \times 1000$$

3.5 Methane estimation by SF_6 tracer technique

Methane production by the animals was measured by the SF_6 tracer technique (Johnson et al., 1994). A permeation tube containing SF_6, an inert gas tracer, was placed into the rumen of each animal 2 days before CH_4 measurement commenced. The permeation tubes were prepared at the Environmental lab, NDRI India, and were filled with a certain amount of SF_6 gas. The average release rate was predetermined over the preceding 40 days period by weighing each permeation tube at the same time point daily for a week and once weekly onwards. A halter fitted with a capillary tube was placed on each animal's mouth and connected to an evacuated sampling canister designed to half fill over a 24-h period. As the vacuum in the sampling canister slowly dissipated, a steady sample of the air around the mouth and nose of the animal was collected. For each day during the measurements, background concentrations of CH_4 and SF_6 were measured by placing one sampling kit (identical to those used on the animals) at a strategic location in the animal house, which was naturally ventilated. The CH_4 and SF_6 concentrations in the gas samples were subsequently adjusted for their background concentrations. After collection of a sample, the canisters were pressurized with nitrogen, and the concentration of SF_6 in the canisters was analyzed by gas chromatography (Nucon 5700, Nucon Engineers, New Delhi), fitted with an electron capture detector (250 ºC) and 3.3 m molecular sieve column with an i.d of 0.32 mm. Another gas chromatograph instrument was fitted with a flame ionization detector (100 ºC) and stainless-steel column packed with Porapak-Q (length 1.5; o.d. 3.2 mm; i.d. 2 mm; mesh range 80-100) to determine CH_4 concentration. The column and injector temperatures were 50 and 40°C in both instruments. All samples were analyzed in duplicate except standards, which were analyzed in triplicate. Nitrogen was used as the carrier gas at a pressure of 1kg/cm2. The standards were run at the beginning and end of each day with the methane standard run every 10 samples throughout the day. Gas concentrations (SF_6 and CH_4) were determined from peak areas and identified from their different retention times relative to the known standards. The methane output calculated using the following formula:

$$CH_4\ (g/d) = \left(\frac{SCH4 - BCH4}{SSF6 - BSF6}\right) \times \left(\frac{MCH4}{MSF6}\right) \times QSF6 \times 1000$$

Where S_{CH4} and B_{CH4} are methane concentrations in the sample and background canisters (ppm), S_{SF6} and B_{SF6} represent the concentrations of SF_6 in sample and background's canister's (ppt), M_{CH4} and M_{SF6} are the molecular weight of methane and SF_6 (g), respectively and Q_{SF6} represents release rate of SF_6 (mg/d).

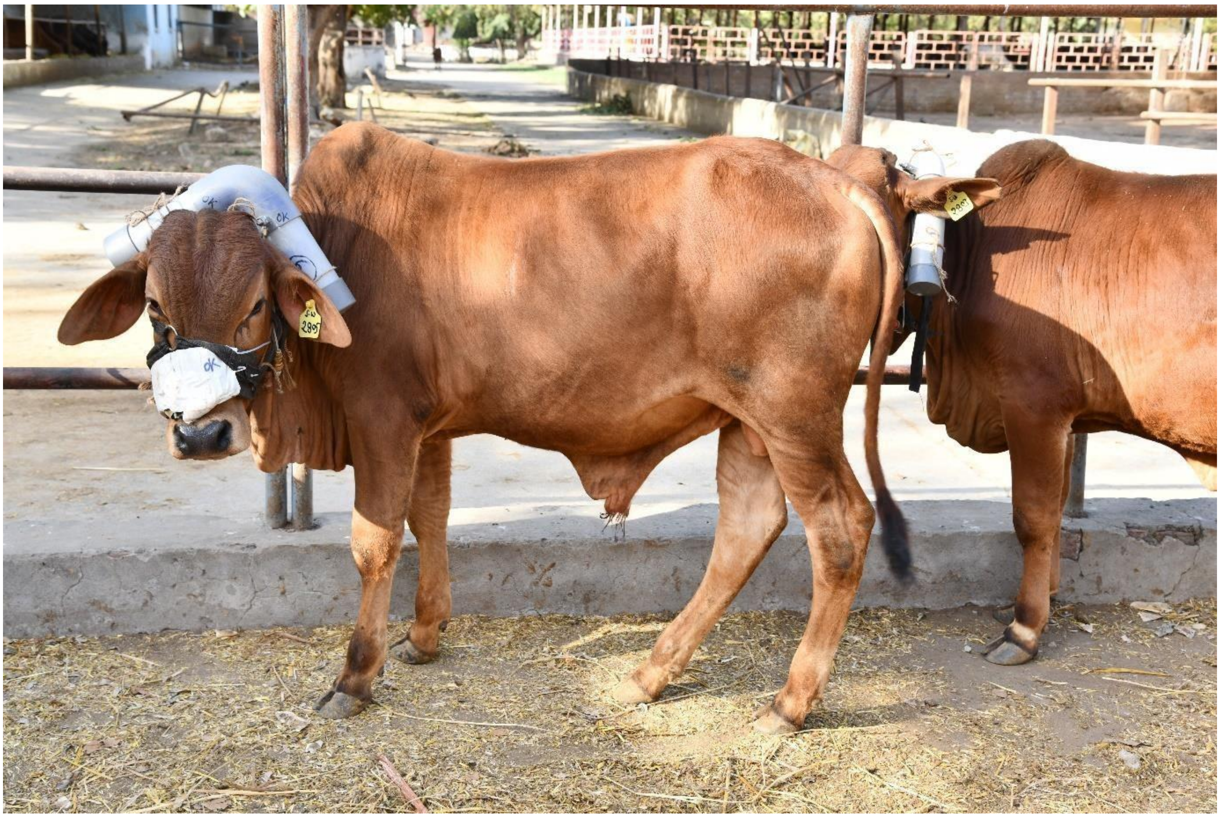

Plate 3.2 Methane estimation from Sahiwal calves during experiment.

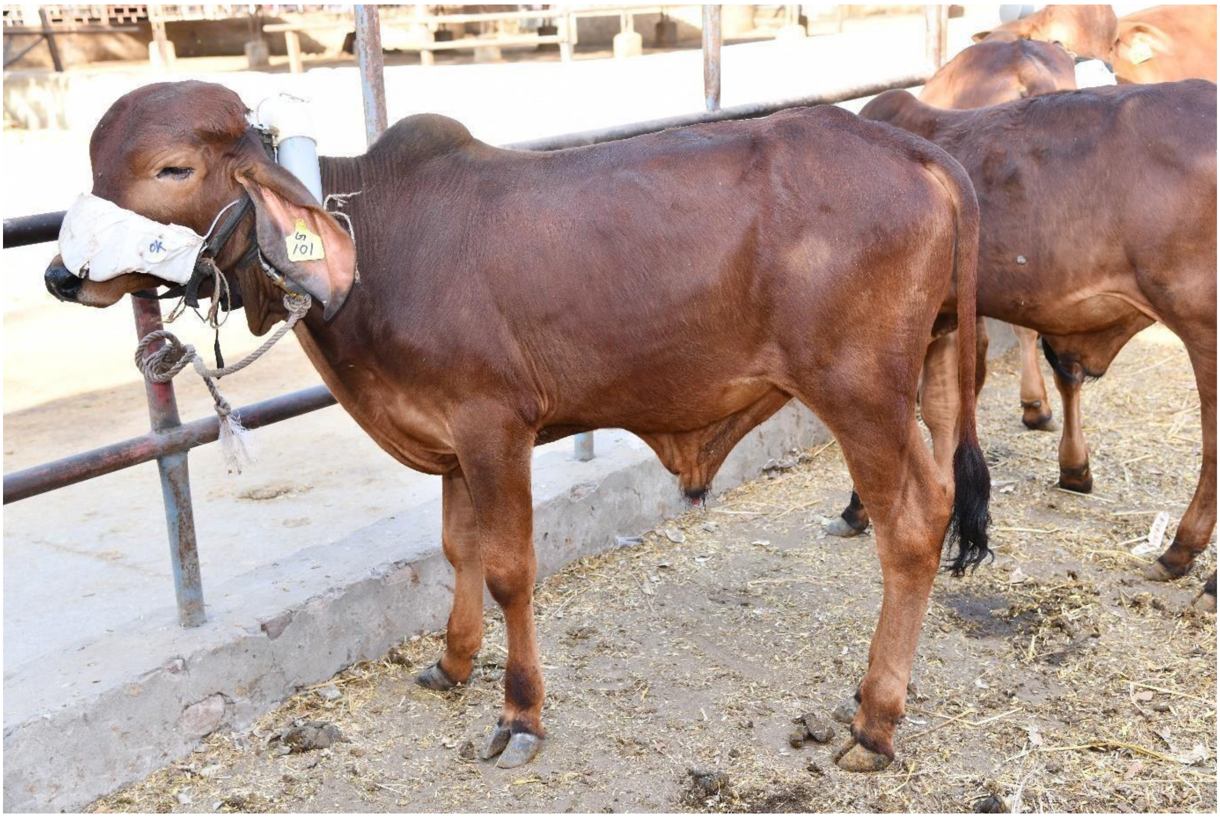

Plate 3.3 Methane estimation from Gir calves during experiment.

3.6 Statistical analysis of experimental data

The data were presented as means with a pooled SE (standard error) for all parameters. Statistical analysis of data for DMI, BW, digestibility, rumen fermentation patterns, and methane emission were carried out by Multi-variate two-factor analysis (ANOVA) using SPSS software version 9.3 (2010).

CHAPTER- 4

RESULTS & DISCUSSION

4.1 Chemical composition of feedstuffs offered to experimental animals during period 1 and period 2

The chemical composition (% DM) of the feedstuffs offered to experimental animals during period 1 and period 2 are presented in table 4.1 and 4.2 respectively. The oat green fodder, wheat straw, and concentrate mixture were having 13.14, 91.00, 88.80% DM in periods1 and 20.08, 90.82, 89.72 in period 2 respectively. There is an increase in DM% of oats fodder in period 2 when compared with period 1, as it implies over the time oats fodder is matured and DM% of fodder increased in period 2. The CP content of oats, WS, and concentrate mixture were 9.46, 3.22, 19.53 in period 1 and 8.07, 3.93, 19.11 (%) in period 2 respectively. The EE content was 2.69, 1.04, 3.19 in period 1 and 2.58, 1.69, 4.46 in period 2 respectively. There was not much variation. The NDF content of oat green fodder, WS, concentrate mixture was 54.97, 73.31, 23.42 in period 1 and 63.75, 82.54, 23.49 in period 2, and ADF content was 39.18, 53.64, 14.84 in period 1 and 40.92, 57.03, 13.51 in period 2 respectively. There is an increase in DM (%) of oats fodder in period 2 when compared with period 1, as it implies dry matter (DM) content increases with advancing the age of fodder (Azim *et al*., 1989). Whereas CP, EE, NDF, and ADF are within the range when period 1 and 2 are compared.

Table 4.1 Chemical composition (% DM basis) of feedstuffs offered to experimental animals during period 1

Parameter	**Oats**	**WS**	**Concentrate mixture**
DM	13.14	91	88.80
OM	90.18	88.15	89.36
CP	9.46	3.22	19.53
EE	2.69	1.04	3.19
NDF	54.97	73.31	23.42

ADF	39.18	53.64	14.84
CF	25.76	37.13	3.74
TA	9.82	11.85	10.64

Table 4.2 Chemical composition (% DM basis) of feedstuffs offered to experimentalanimals during period 2

Parameter	**Oats**	**WS**	**Concentrate mixture**
DM	20.08	90.82	89.72
OM	90.65	89.35	89.75
CP	8.07	3.93	19.11
EE	2.58	1.69	4.46
NDF	63.75	82.54	23.49
ADF	40.92	57.03	13.51
CF	28.33	44.18	3.73
TA	9.35	10.65	10.25

4.2 Plane of nutrition in different groups of animals during the experiment period

Body weights and Intake of various nutrients during the experiment are presented in Table 4.3. The bodyweights of Sahiwal (C), Sahiwal (T), Gir (C), Gir (T) are 159.16±14.99, 161.08±3.55, 167.82±19.09, and, 163.05±10.03 respectively. The intake of DMI (kg/d), DMI (kg/100kg body weight), and OM (kg/d) were 4.14±0.38, 2.61±0.04, and 3.71±0.35 in Sahiwal (C); 3.97±0.14, 2.46±0.05, and 3.55±0.13 in Sahiwal (T); 4.48±0.43, 2.70±0.07 and 4.01±0.39 in Gir (C) and 4.18±0.24, 2.57±0.06 and 3.74±0.21 in Gir (T) respectively which was similar among the breeds. Intake of CP (kg/d) and TDN (kg/d) were 0.53±0.02 and 1.58±0.03 in Sahiwal (C) ; 0.52±0.01 and 1.45±0.07 in Sahiwal (T); 0.55±0.02 and 1.64±0.03 in Gir (C) and 0.52±0.02, 1.54±0.05 kg/d in Gir (T) respectively. Furthermore, the intake of EE, NDF, and ADF was

0.13±0.01, 1.99±0.31 and 1.36±0.17 kg/d in Sahiwal (C); 0.12±0.01, 1.87±0.12 and 1.28±0.06 in Sahiwal (T); 0.14±0.01, 2.21±0.35 and 1.51±0.20 in Gir (C) and 0.12±0.01, 1.97±0.13 and 1.33±0.12 in Gir (T) respectively. From the above data, we can conclude that there was no significant difference among control and treatment groups of both Sahiwal and Gir breeds, this implies that supplementation of methane inhibitors (nitrate, linseed oil, and anthraquinone) does not affect the nutrient intake.

Voluntary feed intake is an important criterion that shows a profound impact on the productivity of the animal, Thus whilst evaluating a feed supplement it is customary to ensure that the supplement being tested does not have any adverse effect on DMI. Plane of nutrition in different groups of animals when observed (Table 4.3) it was concluded that there was no significant difference in the nutrients intake in both control and treatment groups of Sahiwal as well as Gir breeds, this implies supplementation of methane inhibitors (nitrate, linseed oil, and anthraquinone) had not affected the nutrients intake and this observation is supported by previous studies, where nitrate fed to sheep at 2% of the concentrate mixture did not reduce feed intake (Pal *et al.,* 2015) and there was no change in DM intake on feeding 26 g nitrate/kg DMI (Zijderveld *et al.,* 2010). Sakthivel *et al.,* (2011) had also not observed any change by nitrate feeding (2 percent of DMI) on DMI and nutrients digestibility though there was about a 15 percent reduction in methane emission in buffaloes.

Sophal *et al.* (2013) studied the effect of potassium nitrate (@5% of DM) as a source of fermentable nitrogen in local yellow cattle fed a basal diet of cassava root chips and fresh cassava foliage. They found that the DM intake was less on the nitrate diet. However, Sophea and Preston (2010) found no effect on live weight gain or DM feed conversion in growing goats with potassium nitrate (@ 0, 2, 4, 6% of DM) supplementation. Voluntary feed intake generally decreased with a high-fat percentage in the diet (Wanapat *et al.,* 2011). Individual oil has its characteristic odor, fatty acid composition, and effect over rumen dry matter intake as well as digestibility. Dry matter intake also depends on the palatability of the offered feed, the passage rate of the feed, and the rumino-reticular motility. Oil, when reaches to rumen it forms a thin layer coating over the fibrous feed particles in the rumen (Jenkin, 2003) thus hampers the microbial attachment which results in the increased feed retention time in the rumen and decreased voluntary intake (Rumen fill) by animals, consequently daily dry matter intake. In contrast, to the present study, there is a decrease in DMI by 24.76 and15.66% when 5.7% of DM linseed oil and extruded linseed supplemented in silage-based diet to dairy cows (Martin *et al.,* 2008).

Four ruminally cannulated, lactating cows were fed a total mixed ration 50:50 forage: concentrate without supplementation (control, CTL), or supplemented with LO at 2, 3, or 4%. and no effect was observed on DM intake (Benchaar *et al.*,2012).

The present results indicated that supplementation of methane production inhibitors had not influenced feed and nutrient intake.

Table 4.3 Plane of nutrition of different groups of animals during the experiment

Parameter	**SAHIWAL(C)**	**SAHIWAL(T)**	**GIR (C)**	**GIR(T)**	**P Value**
Body weight (Kg)	159.16±14.99	161.08±3.55	167.82±19.09	163.05±10.03	0.971
Feed intake (Kg/ d)					
DMI through oats (kg/d)	1.30±0.21	1.27±0.08	1.45±0.20	1.38±0.08	0.864
DMI through WS (kg/d)	0.88±0.16	0.72±0.09	1.07±0.20	0.85±0.14	0.527
DMI through Conc. (kg/d)	1.97±0.03	1.98±0.02	1.96±0.02	1.95±0.02	0.809
DM intake					
Total DMI (kg/d)	4.14±0.38	3.97±0.14	4.48±0.43	4.18±0.24	0.72
DMI (kg/100kgBW)	2.61±0.04	2.46±0.05	2.70±0.07	2.57±0.06	0.072
DMI (g/kgW$^{0.75}$)	92.24±2.04	87.62±2.15	96.30±2.16	91.69±2.17	0.77
Nutrient intake					
OM intake (kg)	3.71±0.35	3.55±0.13	4.01±0.39	3.74±0.21	0.732

OMI (kg/100kgBW)	2.34±0.03	2.20±0.05	2.41±0.06	2.30±0.06	0.071
CPI (kg/d)	0.53±0.02	0.52±0.01	0.55±0.02	0.52±0.02	0.506
CPI (kg/100kgBW)	0.35±0.02	0.32±0.00	0.34±0.03	0.32±0.01	0.723
EE intake (kg)	0.13±0.01	0.12±0.01	0.14±0.01	0.12±0.01	0.238
EEI (kg/100kg BW)	0.08±0.00	0.07±0.01	0.09±0.00	0.07±0.01	0.076
NDF intake (kg)	1.99±0.31	1.87±0.12	2.21±0.35	1.97±0.13	0.804
NDFI (kg/100kg BW)	1.22±0.08	1.16±0.06	1.28±0.08	1.21±0.04	0.615
ADF intake (kg)	1.36±0.17	1.28±0.06	1.51±0.20	1.33±0.12	0.724
ADFI (kg/100kg BW)	0.84±0.03	0.79±0.03	0.89±0.03	0.81±0.04	0.195
CF intake (kg)	0.76±0.19	0.73±0.09	0.87±0.20	0.83±0.06	0.904
CFI (kg/100kg BW)	0.44±0.08	0.45±0.05	0.48±0.08	0.52±0.03	0.833
TDNI (kg/d)	2.52±0.25	2.33±0.15	2.75±0.30	2.50±0.18	0.651
TDNI (kg/100kgBW)	1.58±0.03	1.45±0.07	1.64±0.03	1.54±0.05	0.117

4.3. Plane of nutrition

Parameter	**SAHIWAL(C)**	**SAHIWAL (T)**	**GIR (C)**	**GIR (T)**
DMI (kg/d)	4.14	3.97	4.48	4.18
DMI (kg/d, ICAR)	4.29	4.34	4.46	4.39
DMI as % of ICAR	**95.98**	**91.18**	**100.16**	**95.29**
CPI (g/d)	532.94	516.90	550.57	524.15
CPI (g/d, ICAR)	577.42	577.88	579.81	578.39
CPI as % of ICAR	**92.22**	**89.04**	**94.86**	**90.56**
TDNI (Kg/d)	2.52	2.33	2.75	2.50
TDNI (g/d, ICAR)	2.34	2.34	2.42	2.36
TDNI as % of ICAR	106.90	99.72	112.43	105.91

The comparison of nutrient intake with that of ICAR (2013) feeding standards isfurnished in Table 4.4. DMI was deficit only by 4.02, 8.82, 4.41 in Sahiwal (C), Sahiwal (T), and Gir (T) respectively, and was excess by 0.16% in Gir (C). The intake of CP was deficit by 7.78, 10.96, 5.14, and 9.44 in Sahiwal (C), Sahiwal (T), Gir (C), and Gir (T) respectively. Similarly, TDN intake was in excess by 6.90, 12.43, 5.91% in Sahiwal (C), Gir (C), and Gir (T)respectively, and deficit by 0.28% in Sahiwal (T). From the above data, we can conclude that there was no significant difference in DMI, CPI by the animals in all the groups when comparedwith ICAR (2013) feeding standards, whereas TDNI was more than ICAR (2013) feeding standards in Gir (C) this may be due to slight increased DMI in this group.

Table 4.4 Nutrients intake by the animals as compared to ICAR Feeding Standard

4.4. Apparent digestibility (%) of nutrients

The digestibility coefficients of DM, OM, CP, EE, NDF, and ADF are presented in Table 4.4. The digestibility coefficients of DM, OM, CP, EE, NDF, and ADF within the Sahiwal breed (Sahiwal (C) 63.19±1.29, 64.31±1.26, 63.46±1.23, 74.60±0.81, 55.46±1.85, 42.98±2.14 and Sahiwal (T) 61.40±1.75, 62.60±1.71, 63.37±1.14, 73.25±1.04, 54.43±2.08, 41.47±2.57 respectively) and in Gir breed (Gir (C) 64.28±1.20, 64.80±1.26, 63.32±0.92, 75.30±1.13, 57.06±2.06, 42.97±2.59 and Gir (T) 62.99±2.15, 63.77±2.13, 62.84±1.94, 73.69±1.51, 54.66±3.26, 40.90±3.55 respectively). From the above data we can conclude that there was no significant change in the apparent digestibility in both control and treatment groups of Sahiwal and Gir breeds, this implies supplementation of methane inhibitors (nitrate, linseed oil, and anthraquinone) does not affect the apparent digestibility.

Table 4.5 Digestibility coefficients (%) of various nutrients in the different groups of animals

Parameter	SAHIWAL(C)	SAHIWAL(T)	GIR (C)	GIR(T)	P Value
DM	63.19±1.29	61.40±1.75	64.28±1.20	62.99±2.15	0.689
OM	64.31±1.26	62.60±1.71	64.80±1.26	63.77±2.13	0.812
CP	63.46±1.23	63.37±1.14	63.32±0.92	62.84±1.94	0.989
EE	74.60±0.81	73.25±1.04	75.30±1.13	73.69±1.51	0.613
NDF	55.46±1.85	54.43±2.08	57.06±2.06	54.66±3.26	0.867
ADF	42.98±2.14	41.47±2.57	42.97±2.59	40.90±3.55	0.934

The digestibility coefficients of DM, OM, CP, EE, NDF, and ADF were estimated, and these did not differ for any parameter among the four groups. No difference in apparent digestibility of nutrients between the control and treatment groups of both breeds is in agreement with the findings of Benchaar *et al*. (2012) who observed the effect of linseed oil (LO) supplementation on nutrient digestibility by supplementation of LO at 2, 3, or 4%.in the diet of lactating cows did not affect DM intake and apparent total-tract digestibility of nutrients. Nitrate and saponin, either alone or in combination also did not reduce the digestibility of DM or NDF (Patra et al., 2014). Whole tract or ruminal DM digestibility (56.8 vs 59.4%), microbial crude protein flow (73.9 vs 58.4 g/day) when sheep fed to a diet consisted of chaffed oat hay supplemented with either 4 or 0% KNO_3 was also not affected (Nolan *et al.*, 2010). Silivong *et al.* (2011) compared the effect of calcium nitrate (3.8% of DM) and sulfur (0.8%) as sodium sulfate on digestibility and nitrogen balance in crossbred goats (*Bach thao* x local female) fed on a basal diet of molasses and Mimosa (*Mimosa piga*) foliage. Digestibility of crude protein and N retention increased by sulphate supplementation, whereas, remained unaffected by nitrate.

Guyader *et al.* (2015) studied the effect of linseed oil and nitrate fed alone or in combination on diet digestibility in cows. The diets were measured in DM. 1) control (CON 50 percent natural grassland hay and 50 percent concentrate), 2) control with 4% linseed oil (LIN), 3) control with 3% calcium nitrate (NIT), and 4) control with 4% linseed oil with 3% calcium nitrate (LIN+NIT). Except for linseed oil, which tended to diminish (P 0.10) fibre digestibility, diets had no effect on total tract digestibility of nutrients (P >0.05).

Gonthier *et al.* (2004) found that supplementation of 3.5 to 4 percent FA from extruded linseed to a grass and corn silage-based diet increased the total digestibility of OM and fiber. The number of additional lipids and their mode of presentation (oil vs. seed) are important determining variables for the unfavorable effect of linseed FA on digestibility, according to the results of these trials. Based on the results of a study by Ben Salem *et al.* (1993) in which cows were fed a meal containing 7% rapeseed oil, they claimed that the negative effect of lipids on digestion is more severe with corn silage diets than with hay diets. Around 90% of total digestible fibre in ruminants is digested in the rumen, while ruminal fibre digestion can be somewhat compensated for by digestion in the large intestine. This matches previous findings on several lipid sources, including linseed oil in cows (Ueda *et al.*, 2003), sheep (Ikwuegbu and Sutton, 1982), and lambs (Machmüller *et al.*, 2000). Kung et al. (2003); Ebrahimi *et al*. (2011) observed that in a digestion trial AQ did not affect the apparent digestibility of nutrients (OM, ADF, NDF, and N) in the total gastrointestinal tract.

4.5 Rumen fermentation patterns in the different groups of animals

Rumen fermentation patterns are presented in table 4.5. The acetate, propionate, and butyrate values were 63.91±0.86, 19.24±0.38, and 12.30±0.29 in Sahiwal (C); 62.9±0.60, 21.86±0.47, and 11.23±0.40 in Sahiwal (T); 62.79±0.65, 18.75±0.37, and 12.71±0.37 in Gir (C) and 61.69±0.21, 21.34±0.18, and 11.68±0.29 in Gir (T) respectively. There was no significant change in acetate, but an increase in propionate and decrease in butyrate values was observed in treatment groups of Sahiwal and Gir when compared with that of control groups. The acetate: propionate ratio was 3.33±0.11, 2.89±0.08, 3.35±0.07, and 2.89±0.01 in Sahiwal (C), Sahiwal (T), Gir (C), and Gir (T) respectively. Acetate: propionate ratio significantly decreased in the treatment groups of both breeds. The NH_3N and Total N were 18.10±0.21 and 72.33±3.90 in Sahiwal (C); 19.88±0.36 and 74.66±1.47 in Sahiwal (T); 19.69±0.48 and 109.66±5.32 in Gir (C) and 21.65±0.94 and 112.00±5.11 in Gir (T) respectively. NH_3N and Total N increased significantly in treatment groups fed with a combination of methane inhibitors (nitrate, linseed oil, and anthraquinone). The pH values were 6.58±0.02, 6.57±0.03, 6.61±0.03, and 6.63±0.04 in Sahiwal (C), Sahiwal (T), Gir (C), and Gir (T) respectively. There was no significant difference in pH of rumen liquor in the control and treatment groups of both breeds.

Table 4.6 Rumen fermentation patterns in the different groups of animals

Parameter	**SAHIWAL(C)**	**SAHIWAL(T)**	**GIR (C)**	**GIR(T)**	**P VALUE**
Acetate	63.91±0.86	62.9±0.60	62.79±0.65	61.69±0.21	0.133
Propionate	19.24^{a}±0.38	21.86^{b}±0.47	18.75^{a}±0.37	21.34^{b}±0.18	<0.01
Butyrate	12.30^{ab}±0.29	11.23^{a}±0.40	12.71^{b}±0.37	11.68^{ab}±0.29	0.03
Acetate: Propionate	3.33^{b}±0.11	2.89^{a}±0.08	3.35^{b}±0.07	2.89^{a}±0.01	<0.01
NH_3N	18.10^{a}±0.21	19.88^{ab}±0.36	19.69^{ab}±0.48	21.65^{b}±0.94	0.03
TOTAL N	72.33^{a}±3.90	74.66^{a}±1.47	109.66^{b}±5.32	112.00^{b}±5.11	<0.01
pH	6.58±0.02	6.57±0.03	6.61±0.03	6.63±0.04	0.480

^{a,b,} bearing different superscripts in the same row differ significantly ($p<0.05$)

Rumen fermentation pattern was observed, there was no significant change in rumen parameters like acetate and pH, whereas there was a significant increase in propionate, ammonia nitrogen, total nitrogen, and a significant decrease in butyrate %.

Nitrate feeding can affect ruminal fermentation in an unadapted animal (Guo *et al.,* 2009) while this effect will disappear when the animal is adapted to dietary nitrate (Zhou *et al.,* 2012).

The increase in propionate may be due to availability of lipids from LO through a shift of fermentation towards propionate at the expense of acetate, and/or by a decrease in protozoans which are hydrogen producers mitigation of rumen methanogenesis can be achieved through a decrease in the hydrogen supply to methanogens, (Morgavi *et al.,* 2010), and also the death of methanogens due to direct toxicity of nitrate and nitrite (Zijderveld *et al*., 2011), and methane production is inhibited by AQ by blocking MCR enzyme (Arokiyaraj *et al*., 2019), and linseed oil supplementation also have a toxic effect on protozoa (Guyader *et al*., 2015), which harbor methanogens. Guyader et al. (2015) studied the effect of linseed oil and nitrate fed alone or in combination on rumen fermentation patterns in cows, where linseed oil diet increased ($P = 0.02$) propionate proportion and decreased ($P = 0.03$) ruminal protozoa concentration compared with CON diet.

The nitrate addition can increase ammonia concentrations in the rumen through respiratory nitrate ammonification (Sar *et al.,* 2005), and ammonia inhibits methanogens at high concentrations (Chen *et al.,* 2007), increasing ammonia concentrations could have also contributed to methane inhibition. In contrast to this study, supplementing rumen-fistulated steers' diets with nitrate at 1 and 2 percent of DM had no effect on ruminal ammonia nitrogen (Zhao *et al.,* 2015).

In another study, the acetate-to-propionate ratio was lowest at the AQ level of 4 ppm because the concentration of propionate increased without a significant change in acetate concentration and had no effect on pH or ammonia nitrogen under in vitro and in vivo conditions, whereas the highest dose of AQ (8 ppm) depressed the concentrations of total VFAs, propionate, valerate, acetate, and ionised calcium (Ebrahimi *et al.,* 2011).

The stimulation of hydrogen utilization towards pathways producing alternative end products such as propionate, which is beneficial for the animal in the absence of a lesser number and lesser activity of methanogens is an ideal goal for achieving desirable responses from methane reduction. Therefore, coupling nitrate, linseed oil, and anthraquinone were considered and found effective.

The decrease in butyrate level in this study is supported by another study where feeding of nitrate (2.6% of DMI) did not affect VFA concentrations except for a decrease in branched-chain fatty acids with a 32 percent reduction in methane production (Zijderveld et al., 2010).

In contrast to the present study total, VFAs and acetate concentration increased linearly by increasing nitrate levels in the diet of nitrate-adapted ruminants (Zhao et al., 2015) which might be due to the level of fiber in the diet.

4.6. Enteric methane emission in animals

Enteric methane (CH_4) emission and related parameters of experimental animals are presented in Table 4.6. CH_4 emission (g/d) was tended to be lower in treatment groups fed with methane inhibitors (nitrate, linseed oil, and anthraquinone combination) (51.90±2.88 in Sahiwal calves, 61.38±1.43 in Gir calves) as compared to groups fed with control diet (65.39±2.70 in Sahiwal, 74.55±1.01 in Gir). No significant ($P>0.05$) changes were seen in CH_4 g/kg DMI, DDMI, OMI, DOMI, NDFI, DNDFI, ADFI, DADFI. The values of methane emission per kg DMI and DDMI ranged between 13-17g and 21 to 27 g respectively in the four groups. The values per kg OMI and DOMI ranged between 14-19 and 23-30g respectively.

Methane emission (g/kgCPI, DCPI) were significantly ($P<0.05$) higher in groups fed with the control diet, Sahiwal (C) 123.63±7.24 CH_4 g/kg CPI, 194.60±10.39 CH_4 g/kg DCPI; Gir (C) 135.92±3.19 CH_4 g/kg CPI, 214.92±6.07 CH_4 g/kg DCPI than the treatment group of Sahiwal (T) 100.64±6.02 CH_4 g/kg CPI, 158.73±8.88 CH_4 g/kg DCPI; Gir (T) 118.20±6.45 CH_4 g/kg CPI, 188.72±10.62 CH_4 g/kg DCPI. Intake of energy (GEI, DEI, MEI) was not significantly different in groups fed with control diet (Sahiwal (C) GEI 76.15±6.30, DEI 45.90±2.97, MEI (MJ/d) 38.12±2.49; Gir (C) GEI 82.18±7.01, DEI 48.78±3.40, MEI 40.56±2.86) when compared with treatment groups fed with methane inhibitors (Sahiwal (T) GEI 73.14±2.56, DEI 44.51±1.21, MEI (MJ/d) 36.93±1.02; Gir (T) GEI 77.02±3.84, DEI 46.26±1.90, MEI 38.40±1.61) because there was no effect on intake as well as digestibility of the feeds in all the four groups.

CH_4 energy (MJ/d) is significantly more in control groups (Sahiwal (C) 3.60±0.14, Gir (C) 4.10±0.05) as compared to their treatment groups (Sahiwal (T) 2.85±0.14, Gir (T) 3.38±0.07). CH_4 energy loss as DEI (%) was significantly ($P<0.05$) higher in control groups (Sahiwal (C) 8.08±0.65, Gir (C) 8.63±0.53, when compared with treatment groups supplemented with methane inhibitors (Sahiwal (T) 6.44±0.36, Gir (T) 7.39±0.41. There is no significant difference in methane energy loss as % GEI, but the decreasing trend was observed for methane energy loss as % MEI in treatment groups when compared with control.

The CH_4 (g/d) was lower in treatment groups of both the species Sahiwal (20.63%) and Gir (17.66%) due to supplementation with the combination of methane inhibitors as compared to their control groups. CH_4 energy loss as GEI, DEI, and MEI was nearly 20%, lower in Sahiwal (T) and 14% in Gir (T) as compared to respective controls. Gir calves were having more CH_4 g/day (12.28%), CH_4 g/kg CPI (9.04%), CH_4E loss as GEI (4.80%), CH_4E loss as DEI(6.37%), CH_4E loss as MEI (6.26%) than the control group of Gir(C). The Gir calves were having more energy loss as methane than Sahiwal calves which showed that treatment effect was more seen in Sahiwal calves as compared to Gir calves.

Table 4.7. Enteric methane emission and energy losses as methane in the four groups

Parameter	**SAHIWAL(C)**	**SAHIWAL(T)**	**GIR (C)**	**GIR(T)**	**P Value**
CH_4 (g/d)	65.39^{b}±2.70	51.90^{a}±2.88	74.55^{c}±1.01	61.38^{b}±1.43	<0.01
CH_4 (g/kg DMI)	16.55±1.72	13.18±0.86	17.37±1.54	14.96±1.06	0.162
CH_4 (g/kg DDMI)	26.24±2.79	21.58±1.56	27.22±2.84	23.90±1.80	0.340
CH_4 (g/kg OMI)	18.50±1.95	14.72±0.96	19.44±1.78	16.70±1.14	0.163
CH_4 (g/kg DOMI)	28.83±3.12	23.61±1.68	30.27±3.28	26.33±1.92	0.313
CH_4 (g/kg CPI)	123.63^{ab}±7.24	100.64^{a}±6.02	135.92^{b}±3.19	118.20^{ab}±6.45	0.04
CH_4 (g/kg DCPI)	194.60^{ab}±10.39	158.73^{a}±8.88	214.92^{b}±6.07	188.72^{ab}±10.62	0.03

CH$_4$ (g/kg NDFI)	37.31±5.85	28.36±2.32	38.53±6.19	31.93±2.39	0.381
CH$_4$ (g/kg DNDFI)	67.92±11.13	52.69±4.86	69.07±13.18	59.96±6.38	0.595
CH$_4$ (g/kg ADFI)	52.32±6.74	41.11±3.04	54.00±7.11	48.64±5.19	0.416
CH$_4$ (g/kg DADFI)	123.61±16.89	102.36±11.68	131.27±25.68	125.26±19.23	0.728
CH$_4$ (g/kg TDNI)	27.39±2.96	22.59±1.59	28.78±3.06	25.14±1.77	0.324
CH$_4$E (MJ/d)	3.60^{b}±0.14	2.85^{a}±0.14	4.10^{c}±0.05	3.38^{b}±0.07	<0.01
GEI (MJ/d)	76.15±6.30	73.14±2.56	82.18±7.01	77.02±3.84	0.736
DEI (MJ/d)	45.90±2.97	44.51±1.21	48.78±3.40	46.26±1.90	0.744
MEI (MJ/d)	38.12±2.49	36.93±1.02	40.56±2.86	38.40±1.61	0.738
CH$_4$ energy loss as %					
GE intake	4.95±0.47	3.93±0.23	5.20±0.41	4.46±0.28	0.14
DE intake	8.08^{ab}±0.65	6.44^{a}±0.36	8.63^{b}±0.53	7.39^{ab}±0.41	0.057
ME intake	9.73±0.79	7.76±0.44	10.38±0.65	8.91±0.50	0.062

^{a,b,c} bearing different superscripts in the same row differ significantly ($p<0.05$)

Methane emissions were significantly low in treatment groups fed with methane inhibitors (nitrate, linseed oil, and anthraquinone) along with control diet when compared with control groups. The decrease in methane is due to the reduction of nitrate (ΔG=-163 KJ mol) and sulfate (ΔG=152 KJ mol) is thermodynamically favored over carbon dioxide reduction (Δ G=130 KJ/mol), which provides nitrate and sulfate-reducing bacteria a competitive advantage over methanogens as hydrogen sink (Oremland et al., 1988). The effects of nitrate (0, 2, 4, and 6% potassium nitrate of DMI) had shown strong inhibition of methanogens (Sophia et al., 2010), and the number of methanogens decreased when nitrate was included in the diet (P<0.001)

(Zijderveld et al., 2010). This is probably the reason for the reduction in methane production in the treatment group of both species.

The inclusion of vegetable oil or lipid is also one of the most promising techniques for reducing methane emission for ruminants (Beauchemin et al., 2008). The addition of fats or oils in the diets of ruminants suppresses methane production by two mechanisms. Fatty acid toxicity to methanogens is the direct mechanism and the indirect mechanism includes protozoal inhibition, reduction of double bonds in unsaturated fatty acids, increased productivity, and enhanced propionate production (Guyader *et al*., 2015). These workers studied the effect of linseed oil and nitrate fed alone or in combination on methane emissions in cows. The diets were measured in DM. 1) control (CON 50 percent natural grassland hay and 50 percent concentrate), 2) control with 4% linseed oil (LIN), 3) control with 3% calcium nitrate (NIT), and 4) control with 4% linseed oil with 3% calcium nitrate (LIN+NIT). Linseed oil and nitrate supplementation reduced CH4 emissions (g/kg DMI) by 17 and 22 percent, respectively, when fed alone, and by 32 percent when fed together, when compared to the CON diet.

Rumen methanogens in ruminants use the methanogenesis pathway (Hydrogenotrophic) to convert H_2 and CO_2 (generated by bacteria, protozoa, and anaerobic fungus) into CH_4 (Patra et al., 2010). For this process (methanogenesis), methanogenic archaea require the methyl coenzyme M reductase (MCR) for the formation of methane. AQ has more specificity towards the methyl-coenzyme M reductase binding site and could be a potent methanogen inhibitor (Arokiyaraj et al., 2019). The ability of AQ to impede electron transfer and uncouple Adenosine Triphosphate (ATP) synthesis associated with sulphate reduction was linked to its action. In the final step of methanogenesis, the methyl reductase system in methanogens allows the transfer of electrons, generating a proton motive force of one ATP per two electrons, similar to that of sulfate-reducing bacteria (Garcia et al., 1996). The metabolic route common to all methanogens includes the reductive demethylation of methyl coenzyme M to methane. It was postulated that AQ uncoupled the electron transfer from cytochrome-linked or membrane-bound ATP synthesis, blocking the reduction of methyl coenzyme M to methane, based on the similarities of ATP creation in methanogens and sulfate-reducing bacteria (Garcia *et al.,* 1996).

Hence, looking into the mechanisms involved as expected, a reduction in methane emission was observed in the treatment groups of both breeds.

4.7 Correlation of nutrient intake (kg) and digestible nutrient intake with enteric methane emission (g/d)

A correlation between methane emission (g/d) and various nutrients intake was also estimated and values are presented in table 4.8. It was observed that correlation with DMI (0.69), DDMI (0.57), DOMI (0.58), DNDFI (0.48), DADFI (0.39), and TDNI (0.56) were found positive. In this study correlation with DMI, DDMI, DOMI, and TDNI were strong. Ulyatt and Lassey (2001) were found a stronger negative relationship (r= -0.597) revealed that as intake of nutrients increased the percentage of dietary energy lost as methane decreased. Moss (1993) observed a positive correlation with NDF content (R^2= 79%) and negative relation with CP content (R^2=-76.8%). Mohini and Singh (2001) also reported a significant (P<0.05) positive correlation of methane emission with digestible nutrient intake.

Table 4.8 Correlation of digestible nutrient intake (kg) with enteric methane emission (g/d)

Parameter	Methane emission (g/d)
DMI	0.69
DDMI	0.57
DOM	0.58
DNDF	0.48
DADF	0.39
TDNI	0.56

CHAPTER- 5

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

The experiment was conducted to estimate nutrient digestibility, rumen fermentation patterns, and methane emission in Sahiwal and Gir calves supplemented with a combination of methane inhibitors (nitrate, linseed oil, and anthraquinone). This study was conducted at LRC of NDRI, Karnal. The study consists of a total of 12 healthy 6 Sahiwal calves and 6 Gir calves. The animals were divided into 4 groups of 3 animals in each, based on their average body weight. In the control groups of both the species the animals fed diet as per ICAR 2013 and in treatment groups animals were fed with control diet + combination of methane inhibitors (nitrate, linseed oil, and anthraquinone). The design of the experiment was switch over consisting of period 1 and period 2 each of 60 days. The calves of all experimental groups received oat green fodder, wheat straw, and concentrate mixture. During the last week of each period of the experiment, a digestibility trial of seven days was conducted with a total collection of faeces, residue, and feed to study the digestibility of various nutrients. The methane was estimated by using the SF_6 tracer technique by taking 5 successful collection and rumen fermentation patterns studied by collecting rumen liquor at the end of each period. The summary and conclusion of this study are represented in this chapter.

5.1 Chemical composition of feedstuffs offered to experimental animals during period 1 and period 2

The oat green fodder, wheat straw, and concentrate mixture were having 13.14, 91, 88.80% DM in period 1 and 20.08, 90.82, 89.72 in period 2 respectively. There was an increase in DM% of oats fodder in period 2 when compared with period 1, There was no significant change in CP, EE, NDF, and ADF when period 1 and 2 and the values were in the normal range.

5.2 Plane of nutrition in different groups of animals during the experiment period

The bodyweights of Sahiwal (C), Sahiwal (T), Gir (C), Gir (T) are 159.16±14.99, 161.08±3.55, 167.82±19.09, and, 163.05±10.03 respectively. The intake of DM (kg/d) and OM (kg/d) was 4.14±0.38 and 3.71±0.35 in Sahiwal (C); 3.97±0.14 and 3.55±0.13 in Sahiwal (T); 4.48±0.43, 4.01±0.39 in Gir (C) and 4.18±0.24, 3.74±0.21 in Gir (T) respectively which was similar among the breeds. Intake of CP (kg/d) and TDN (kg/d) were 0.53±0.02 and 1.58±0.03 in

Sahiwal (C) ; 0.52±0.01 and 1.45±0.07 in Sahiwal (T); 0.55±0.02 and 1.64±0.03 in Gir (C) and 0.52±0.02, 1.54±0.05 kg/d in Gir (T) respectively.

5.3. Plane of nutrition

DMI was deficit by 4.02, 8.82, 4.41 in Sahiwal (C), Sahiwal (T), and Gir (T) respectively and 0.16% excess in Gir (C) when compared with ICAR (2013) feeding standards. The intake of CP was deficit by 7.78, 10.96, 5.14, and 9.44 in Sahiwal (C), Sahiwal (T), Gir (C), and Gir (T) respectively. Similarly, TDN intake was excess by 6.90, 12.43, 5.91% in Sahiwal (C), Gir (C), and Gir (T) respectively, and deficit by 0.28% in Sahiwal (T). From the above data we can conclude that there’s not much difference in DMI, CPI of animals when compared with ICAR (2013) feeding standards, whereas TDNI is excess than ICAR (2013) feeding standards in Gir (C) this may be due to slight increased DMI in this group.

5.4. Apparent digestibility (%) of nutrients

The digestibility coefficients of DM, OM, CP, EE, NDF, and ADF within Sahiwal (C) 63.19±1.29, 64.31±1.26, 63.46±1.23, 74.60±0.81, 55.46±1.85, 42.98±2.14 and Sahiwal (T) 61.40±1.75, 62.60±1.71, 63.37±1.14, 73.25±1.04, 54.43±2.08, 41.47±2.57 respectively and in Gir breed (C) 64.28±1.20, 64.80±1.26, 63.32±0.92, 75.30±1.13, 57.06±2.06, 42.97±2.59 and Gir (T) 62.99±2.15, 63.77±2.13, 62.84±1.94, 73.69±1.51, 54.66±3.26, 40.90±3.55 respectively did not differ among the groups.

5.5 Rumen fermentation patterns in the different groups of animals

The acetate, propionate, and butyrate values were 63.91±0.86, 19.24^{a}±0.38, and 12.30±0.29 in Sahiwal (C); 62.9±0.60, 21.86±0.47, and 11.23±0.40 in Sahiwal (T); 62.79±0.65, 18.75±0.37, and 12.71±0.37 in Gir (C) and 61.69±0.21, 21.34±0.18, and 11.68±0.29 in Gir (T) respectively. The acetate: propionate ratio is 3.33±0.11, 2.89±0.08, 3.35±0.07, and 2.89±0.01 in Sahiwal (C), Sahiwal (T), Gir (C), and Gir (T) respectively. The NH_3N and Total N were 18.10±0.21 and 72.33±3.90 in Sahiwal (C); 19.88±0.36 and 74.66±1.47 in Sahiwal (T); 19.69±0.48 and 109.66±5.32 in Gir (C) and 21.65±0.94 and 112.00±5.11 in Gir (T) respectively. NH_3N and Total N increased significantly in treatment groups fed with a combination of methane inhibitors (nitrate, linseed oil, and anthraquinone). The pH was 6.58±0.02, 6.57±0.03, 6.61±0.03, and 6.63±0.04 in Sahiwal (C), Sahiwal (T), Gir (C), and Gir (T) respectively.

5.6. Enteric methane emission in animals

CH_4 emission (g/d) tended to be lower in treatment groups fed with methane inhibitors (nitrate, linseed oil, and anthraquinone combination) (51.90±2.88 in Sahiwal treatment calves, 61.38±1.43 in Gir treatment calves) as compared to groups fed with control diet (65.39±2.70 in Sahiwal control, 74.55±1.01 in Gir control). Methane emission (g/kgCPI, DCPI) were significantly ($P<0.05$) higher in groups fed with control diet Sahiwal (C) 123.63±7.24 CH_4 g/kg CPI, 194.60±10.39 CH_4 g/kg DCPI; Gir (C) 135.92±3.19 CH_4 g/kg CPI, 214.92±6.07 CH_4 g/kg DCPI) then the treatment group of Sahiwal (T) 100.64±6.02 CH_4 g/kg CPI, 158.73±8.88 CH_4 g/kg DCPI; Gir (T) 118.20±6.45 CH_4 g/kg CPI, 188.72ab±10.62 CH_4 g/kg DCPI). No significant changes were seen in CH4 g/kg DMI, DDMI, OMI, DOMI, NDFI, DNDFI, ADFI, DADFI ($P>0.05$).

CH_4 energy (MJ/d) is significantly more in control groups (Sahiwal (C) 3.60±0.14, Gir (C) 4.10±0.05) as compared to their treatment groups (Sahiwal (T) 2.85±0.14, Gir (T) 3.38±0.07). CH_4 energy loss as DEI was significantly ($P<0.05$) higher in control groups (Sahiwal (C) 8.08±0.65 as DEI, Gir (C) 8.63±0.53 as DEI, when compared with treatment groups supplemented with methane inhibitors Sahiwal (T) 6.44±0.36 as DEI, Gir (T) 7.39±0.41 as DEI. There is no significant difference in methane energy loss as % GEI, but the decreasing trend of methane energy loss as % MEI in treatment groups, when compared with control.

The CH_4 (g/d) was tended to be 20.63% lower in Sahiwal (T) and 17.66% lower in Gir (T) treatment groups supplemented with the combination of methane inhibitors as compared to their control groups. CH_4 energy loss as GEI, DEI, and MEI was20.60%, 20.29%, and 20.24% lower in Sahiwal (T) group as compared to Sahiwal (C) group and Gir (T) were having 14.23%, 14.36%, and 14.16%less CH4 energy loss as GEI, DEI, and MEI than Gir (T) group. Gir calves were having more (12.28%) CH_4 g/day, (9.04%) CH_4 g/kg CPI, (4.80%) CH_4E loss as GEI, (6.37%) CH_4E loss as DEI, (6.26%) CH_4E loss as MEI than the control group of Gir(C). The Gir calves were having more energy loss as methane than Sahiwal calves which showed that treatment effect was more seen in Sahiwal calves as compared to Gir calves.

5.7 Correlation of nutrient intake (kg) and digestible nutrient intake with enteric methane emission (g/d)

A correlation between methane emission (g/d) and nutrient intake was also estimated and values are presented in table 4.8. it was observed that correlation with DMI (0.69), DDMI (0.57), DOMI (0.58), DNDFI (0.48), DADFI (0.39), and TDNI (0.56) were found positive. In this study correlation with DMI, DDMI, DOMI, and TDNI were strong.

5.8 Conclusions:

Following conclusions were drawn from the results of present experiment.

- The supplementation of combination of methane inhibitors (nitrate, linseed oil, and anthraquinone) has not affected nutrient intake and nutrients digestibility in both the breeds, Sahiwal and Gir calves. It has caused increase in propionate and nitrogen in the rumen.
- CH_4 production decreased by 20.63 % and 17.66 % in the treatment groups of Sahiwal and Gir calves respectively and CH_4E loss (MJ/d) as % DEI was significant. Sahiwal calves produced 12.28 % less CH_4, as compared to Gir calves.

CHAPTER- 6

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