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Evaluation of Rumen Methane Emission in Sahiwal and Gir Calves Supplemented with Combination of Methanogenic Inhibitors

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Abstract: Methane is one of the main greenhouse gases emitted by ruminants around the world. It is essential to investigate novel approaches to increasing animal production while reducing greenhouse gas emissions from ruminants. This study was conducted to examine the effect of methane inhibitors, such as nitrate, linseed oil, and anthraquinone, on nutritional digestibility, rumen fermentation processes, and methane emission in Sahiwal and Gir cattle calves. Twelve calves (6–12 months old), six of each Sahiwal and Gir breed, were selected and divided into four groups; Sahiwal control (C) and treated (T) calves; Gir control (C) and treated calves (T) of three calves each based on average body weight. Switch over a design was used as for periods 1 and 2. Animals in all groups were fed chopped oat fodder, wheat straw, and a concentrate mixture. Additionally, treated groups were fed a ration with potassium nitrate (1%), linseed oil (0.5%), and anthraquinone (4 ppm). The results revealed that the addition of methane inhibitors had no impact on nutrient intake and apparent digestibility. The levels of propionate, ammonia nitrogen, and total nitrogen were increased significantly ($p < 0.05$), while butyrate decreased in the treated groups of both breeds. However, there was no change in acetate and pH between the groups. Methane emission (g/d) was lower ($p < 0.05$) in the treated groups as compared to the control group. This study concludes that supplementation of methane inhibitors in calves feed can be utilized to lower methane emissions without affecting the intake and digestibility of nutrients. Combining diverse dietary mitigation strategies could be an effective way to mitigate methane emissions to reduce global warming while minimizing any negative impacts on ruminants to accomplish sustainable animal production.

Keywords: Gir calves; Sahiwal; methane mitigation; nutrient utilization

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1. Introduction

Livestock is responsible for more than 15% of anthropogenic methane emissions via enteric fermentation. Rumen fermentation in ruminants produces methane, which results in a 2–12% loss of food energy while also contributing to greenhouse gas (GHG) burdens [1]. Ruminal methane is produced by methanogenic archaea by combining CO_2 and hydrogen (H+). Hydrogen must be removed because its buildup hinders a variety of biological processes needed to keep the rumen environment healthy. Methanogenesis appears to be the main pathway for H+ elimination in the rumen, despite the presence of alternative pathways, such as acetogenesis, propionogenesis, sulphate, and nitrate reduction [2]. Ruminant methane emissions also represent precious metabolic energy that is lost to the animals and must be replaced by their diet, which is another issue related to these emissions that goes beyond global warming. Thus, enteric methane reduction is being considered a promising technique for improving ruminant feed efficiency and lowering GHG emissions.

Many natural substances have been investigated to determine their efficacy in lowering methane emission under in vitro and in vivo conditions while optimizing rumen fermentation [3,4]. Utilizing substances that consume hydrogen is one of the most important ways to reduce methanogenesis. Nitrate is a common hydrogen-consuming chemical used to minimize methane production, and a non-protein nitrogen supply for rumen microbes [5]. The application of nitrate along with cysteamine had a positive impact on rumen fermentation while lowering the total gas and methane production by increasing the fiber degrading and hydrogen using microbes [6]. Long-term feeding of coated nitrate has also been shown in vivo to continually lower the intestinal methane emissions of grazing steers [7]. Adding sodium nitrate (0.11–0.44 kg body weight) did not negatively affect the water buffaloes milk yield, fatty acid, and rumen methanogen/*Butyrivibrio* population associated with biohydrogenation; however, it did increase the rumen volatile fatty acid and microbial diversity [8]. Application of nitrate reduced methane production linearly while having moderate impacts on rumen fermentation and have no effect on nutrient digestion in dairy cows [9].

It is also well known that supplemental lipids in the diet have been shown to change the rumen environment, affecting microbial fermentation and *volatile fatty acid* (VFA) synthesis. Linseed is one of the most intriguing lipid supplements in Malaysia and China because of its lower content of linoleic and saturated fatty acids and contains higher levels of alfa-linoleic acid and omega 3-fatty acid [10,11]. These substances have beneficial bioactivity for animal health due to their anti-inflammatory, antioxidative, and lipid-modulating effects [12]. Linseed has been used in ruminant nutrition to increase milk production and its quality [13]. It was observed that supplementing with linseed oil is a viable way to improve the composition of fatty acid in goat milk without affecting the animal performance parameters [14]. A naturally occurring aromatic chemical molecule, such as anthraquinone (9, 10-dioxoanthracene, AQ) is present in several plants. There are various natural sources of AQ, such as *Cassia fistula*, *Aloe succtrina*, *Senna alexandri*, and *Artemisia scoparia*, etc. AQ compounds are employed as laxatives, but they have antibacterial, antiviral, and antiparasitic effects. The rhubarb compounds, such as phthalic acid, isobutyl octadecyl ester, and di iso-octyl phthalate, were shown to have more specificity towards the binding site of methyl-coenzyme M reductase and may be an effective methanogen inhibitor [15]. However, studies on the combined impact of nitrate along with linseed oil and anthraquinone on the alteration of ruminal fermentation parameters are scarce. However, it is difficult to use a single component to impart the National environment program to herders, so a combination may be used. Consequently, the current study was conducted to assess the impact of methanogen inhibitors (nitrate, linseed oil and anthraquinone) on nutritional digestibility, rumen fermentation processes, and methane emission in Sahiwal and Gir calves.

2. Results

2.1. Chemical and Mineral Composition of Feed

The chemical composition (% DM) of feed provided to experimental animals is presented in Table 1. The oat green, wheat straw and concentrate mixture had 16.61, 91.91, and 89.26% of DM, respectively. Oats, wheat straw, and concentrate mixture CP contents were 8.76, 3.57, and 19.32%, respectively. The EE content was 2.63, 1.36, and 3.82%, respectively.

2.2. Body Weight and Intake of Nutrients

The body weight and nutrient intake data during the experiment are depicted in Table 2. Sahiwal (C and T) and Gir (C and T) had no change in body weight (159.16, 161.08, 167.82, and 163.05, respectively). It was observed that the addition of methane inhibitors had no impact on nutrient intake. The dry matter intake (DMI, kg/d) was similar among breeds and was 4.14, 3.97, 4.48, and 4.18 kg/d in Sahiwal (C), Sahiwal (T), Gir (C), and Gir (T), respectively. The CP and TDN intake were 0.53, 1.58 in Sahiwal (C), 0.54 and 1.45 in Sahiwal (T), 0.55, 1.64 in Gir (C), and 0.52 and 1.54 in Gir (T), respectively. In addition,

the daily intake of EE, NDF, and ADF was 0.13, 1.99 and 1.36 kg/d in Sahiwal (C), 0.12, 1.87, and 1.28 in Sahiwal (T), 0.14, 2.21, and 1.51 in Gir (C) and 0.12, 1.97, and 1.33 in Gir (T), respectively.

Table 1. Chemical composition of feedstuffs during the experiment.

Parameter	Oats	Wheat Straw	Concentrate Mixture
Dry matter (%)	16.61	90.91	89.26
Organic matter (%)	90.41	88.75	89.55
Crude protein (%)	8.76	3.57	19.32
Ether extract (%)	2.63	1.36	3.82
Neutral detergent fiber (%)	59.36	77.92	23.45
Acid detergent fiber (%)	40.05	55.33	14.17
Crude fiber (%)	27.04	40.65	3.73
Total ash (%)	9.58	11.25	10.44

Table 2. Growth performance, nutrient intake, and digestibility in different groups: Sahiwal control (C), Sahiwal treated (T), Gir control (C), and Gir treated calves (T).

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
Dry Matter intake through oats (kg/d)	1.30 ± 0.21	1.27 ± 0.08	1.45 ± 0.20	1.38 ± 0.08	0.864
Dry matter intake through wheat straw (kg/d)	0.88 ± 0.16	0.72 ± 0.09	1.07 ± 0.20	0.85 ± 0.14	0.527
Dry matter intake through Concentrate mix. (kg/d)	1.97 ± 0.03	1.98 ± 0.02	1.96 ± 0.02	1.95 ± 0.02	0.809
Total dry matter intake (kg/d)	4.14 ± 0.38	3.97 ± 0.14	4.48 ± 0.43	4.18 ± 0.24	0.720
Dry matter intake (kg/100 kg BW)	2.61 ± 0.04	2.46 ± 0.05	2.70 ± 0.07	2.57 ± 0.06	0.072
Dry matter intake (g/kgW $^{0.75}$)	92.24 ± 2.04	87.62 ± 2.15	96.30 ± 2.16	91.69 ± 2.17	0.770
Organic matter intake (kg)	3.71 ± 0.35	3.55 ± 0.13	4.01 ± 0.39	3.74 ± 0.21	0.732
Organic matter intake (kg/100 kg BW)	2.34 ± 0.03	2.20 ± 0.05	2.41 ± 0.06	2.30 ± 0.06	0.071
Crude protein intake (kg/d)	0.53 ± 0.02	0.52 ± 0.01	0.55 ± 0.02	0.52 ± 0.02	0.506
Crude protein intake (kg/100 kg BW)	0.35 ± 0.02	0.32 ± 0.00	0.34 ± 0.03	0.32 ± 0.01	0.723
Ether extract intake (kg)	0.13 ± 0.01	0.12 ± 0.01	0.14 ± 0.01	0.12 ± 0.01	0.238
Ether extract intake (kg/100 kg BW)	0.08 ± 0.00	0.07 ± 0.01	0.09 ± 0.00	0.07 ± 0.01	0.076
Neutral detergent fiber intake (kg)	1.99 ± 0.31	1.87 ± 0.12	2.21 ± 0.35	1.97 ± 0.13	0.804
Neutral detergent fiber (kg/100 kg BW)	1.22 ± 0.08	1.16 ± 0.06	1.28 ± 0.08	1.21 ± 0.04	0.615
Acid detergent fiber intake (kg)	1.36 ± 0.17	1.28 ± 0.06	1.51 ± 0.20	1.33 ± 0.12	0.724
Acid detergent fiber (kg/100 kg BW)	0.84 ± 0.03	0.79 ± 0.03	0.89 ± 0.03	0.81 ± 0.04	0.195
Crude fiber intake (kg)	0.76 ± 0.19	0.73 ± 0.09	0.87 ± 0.20	0.83 ± 0.06	0.904
Crude fiber intake (kg/100 kg BW)	0.44 ± 0.08	0.45 ± 0.05	0.48 ± 0.08	0.52 ± 0.03	0.833
Total digestible nutrients intake (kg/d)	2.52 ± 0.25	2.33 ± 0.15	2.75 ± 0.30	2.50 ± 0.18	0.651
Total digestible nutrients intake (kg/100 kg BW)	1.58 ± 0.03	1.45 ± 0.07	1.64 ± 0.03	1.54 ± 0.05	0.117

Values in tables were the means ± standard.

2.3. Apparent Digestibility Coefficients of Nutrients

The digestibility coefficients of DM, OM, CP, EE, NDF, and ADF were 63.19, 64.31, 63.46, 74.60, 55.46, and 42.98% in the control calves, 61.40, 62.60, 63.37, 73.25, 54.43, and 41.47 in treated Sahiwal calves, 62.28, 64.80, 63.32, 75.30, 57.06, and 42.97 in control and 62.99, 63.77, 62.84, 73.69, 54.66, and 40.90 in treated Gir calves, respectively (Table 3). It was observed that there was no discernible change in the apparent digestibility of the Sahiwal and Gir breeds in either control or treated groups.

Table 3. Digestibility coefficients (%) of various nutrients in the different groups of calves: Sahiwal control (C), Sahiwal treated (T), Gir control (C), and Gir treated calves (T).

Parameter	Sahiwal (C)	Sahiwal (T)	Gir (C)	Gir (T)	*p*-Value
Dry matter (%)	63.19 ± 1.29	61.40 ± 1.75	64.28 ± 1.20	62.99 ± 2.15	0.689
Organic matter (%)	64.31 ± 1.26	62.60 ± 1.71	64.80 ± 1.26	63.77 ± 2.13	0.812
Crude protein (%)	63.46 ± 1.23	63.37 ± 1.14	63.32 ± 0.92	62.84 ± 1.94	0.989
Ether extract (%)	74.60 ± 0.81	73.25 ± 1.04	75.30 ± 1.13	73.69 ± 1.51	0.613
Neutral detergent fiber (%)	55.46 ± 1.85	54.43 ± 2.08	57.06 ± 2.06	54.66 ± 3.26	0.867
Acid detergent fiber (%)	42.98 ± 2.14	41.47 ± 2.57	42.97 ± 2.59	40.90 ± 3.55	0.934

Values in tables were the means ± standard.

2.4. Rumen Fermentation Parameters in Different Groups

The levels of acetate, propionate, and butyrate were 63.91, 19.24, and 12.30% in control; 62.90, 21.86, and 11.23% in treated Sahiwal calves; 62.79, 18.75, and 12.71% in control and 61.69, 21.34, and 11.68% in Gir calves treated with methane inhibitors, respectively (Table 4). Acetate values did not vary significantly, although there was an increase in propionate and a decrease in butyrate between the treated groups of Sahiwal and Gir as compared to the control. The ratio of acetate: propionate ($p > 0.05$) was 3.33, 2.89, 3.35, and 2.89 in Sahiwal and Gir calves. In both breeds, treatment with methane inhibitors caused a considerable decline in the A:P ratio. The NH3N (mg/dL) and total N (g/dL) values considerably increased in treated groups. The values for NH3N and N were 18.10 and 72.33 in the control group of Sahiwal calves, 19.88 and 74.66 in treated Sahiwal (T), 19.69 and 109.66 in Gir control calves, and 21.65 and 112.00 in treated Gir calves, respectively.

Table 4. Rumen fermentation patterns in the different groups of calves: Sahiwal control (C), Sahiwal treated (T), Gir control (C), and Gir treated calves (T).

Parameter	Sahiwal (C)	Sahiwal (T)	Gir (C)	Gir (T)	*p*-Value
Acetate (%)	63.91 ± 0.86	62.90 ± 0.60	62.79 ± 0.65	61.69 ± 0.21	0.130
Propionate (%)	19.24^{a} ± 0.38	21.86^{b} ± 0.47	18.75^{a} ± 0.37	21.34^{b} ± 0.18	<0.011
Butyrate (%)	12.30^{ab} ± 0.29	11.23^{a} ± 0.40	12.71^{b} ± 0.37	11.68^{ab} ± 0.29	0.033
A:P	3.33^{b} ± 0.11	2.89^{a} ± 0.08	3.35^{b} ± 0.07	2.89^{a} ± 0.01	<0.013
NH_3N (mg/dL)	18.10^{a} ± 0.21	19.88^{ab} ± 0.36	19.69^{ab} ± 0.48	21.65^{b} ± 0.94	0.034
Total N (g/dL)	72.33^{a} ± 3.90	74.66^{a} ± 1.47	109.66^{b} ± 5.32	112.00^{b} ± 5.11	<0.014
pH	6.58 ± 0.02	6.57 ± 0.03	6.61 ± 0.03	6.63 ± 0.04	0.485

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

2.5. Enteric Methane Emission in Different Groups

Methane emission (g/d) was lower ($p < 0.01$) in the treated groups (51.90 and 61.38 in Sahiwal and Gir, respectively) as compared to the control group (65.39 and 74.55 in Sahiwal and Gir, respectively). Methane emissions per kg dry matter intake (DMI) and digestible dry matter intake (DDMI) ranged between 13–17 g and 21–27 g in the four groups. The organic matter intake (OMI) and digestible dry organic matter intake (DOMI) levels were between 14–19 and 23–30 g, respectively. Methane emissions (g/kg CPI) were significantly ($p < 0.05$) greater in control groups of both breeds Sahiwal C (123.63) and Gir C (135.92) than the treated groups Sahiwal T (100.64) and Gir T (118.20), respectively (Table 5). Due to the addition of methane inhibitors, the CH4 (g/d) was lower in the treated groups as compared to the control groups. The CH4 energy loss as GEI, DEI, and MEI was less than 20% and 14% in Sahiwal T and Gir T compared to the control groups. Gir calves lost more energy as methane as compared to Sahiwal, indicating that Sahiwal calves had a greater response toward the methane inhibitor supplementation.

Table 5. Rumen methane emission in different groups: Sahiwal control (C), Sahiwal treated (T), Gir control (C), and Gir treated calves (T).

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 (−20.63%)	74.55^{c} ± 1.01 (+12.28%)	61.38^{b} ± 1.43 (−17.66%)	<0.010
CH4 (g/kg DMI)	16.55 ± 1.72	13.18 ± 0.86 (−20.36%)	17.37 ± 1.54 (+4.72%)	14.96 ± 1.06 (−13.87%)	0.162
CH4 (g/kg DDMI)	26.24 ± 2.79	21.58 ± 1.56 (−17.76%)	27.22 ± 2.84 (+3.60%)	23.90 ± 1.80 (−12.20%)	0.340
CH4 (g/kg OMI)	18.50 ± 1.95	14.72 ± 0.96 (−20.43%)	19.44 ± 1.78 (+4.84%)	16.70 ± 1.14 (−14.09%)	0.163
CH_4 (g/kg DOMI)	28.83 ± 3.12	23.61 ± 1.68 (−18.10%)	30.27 ± 3.28 (+4.76%)	26.33 ± 1.92 (−13.01%)	0.313
CH_4 (g/kg CPI)	123.63^{ab} ± 7.24	100.64^{a} ± 6.02 (−18.59%)	135.92^{b} ± 3.19 (+9.04%)	118.20^{ab} ± 6.45 (−13.03%)	0.040
CH_4 (g/kg TDNI)	27.39 ± 2.96	22.59 ± 1.59 (−17.52%)	28.78 ± 3.06 (+4.83%)	25.14 ± 1.77 (−12.65%)	0.324
CH_4 energy loss as %					
Gross energy intake (MJ/d)	4.95 ± 0.47	3.93 ± 0.23	5.20 ± 0.41	4.46 ± 0.28	0.140
Digestible energy intake (MJ/d)	8.08^{ab} ± 0.65	6.44^{a} ± 0.36	8.63^{b} ± 0.53	7.39^{ab} ± 0.41	0.057
Metabolizable energy intake (MJ/d)	9.73 ± 0.79	7.76 ± 0.44	10.38 ± 0.65	8.91 ± 0.50	0.062

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

3. Discussion

3.1. Feed Intake and Digestibility

Sahiwal and Gir are two milch cattle breeds popular in Northern and Western India due to their distinct appearance, ability to withstand high temperatures, and resistance to parasite infestation. This part of the country is characterized by extreme weather conditions, and more than 50% of the human population is dependent on animal protein/other nutrients from milk and milk products. There has been no research conducted to compare the nutrient intake, digestibility, and methane emission in Gir and Sahiwal breed cattle under similar feeding conditions. GHG emission is a global issue, and livestock has a huge impact on a country's GHG inventory making up 60% of the agricultural sector. Therefore, it is crucial to monitor methane emission and rumen fermentation when feeding these popular breeds in India. As India has a diverse population of livestock, enteric methane emissions may not be similar even under similar feeding regimens. Again, combinations of lipids, plant secondary metabolites, and chemical additives have an effect on the mitigation of methane from ruminants. Lipid feeding, such as linseed oil, reduced methane emission by reducing the quantity of OM fermented in the rumen and by exerting direct toxic effects of FA on rumen methanogens [16]. Nitrate may reduce methane emission by competing with methanogenesis for accessible hydrogen in the rumen [9]. AQ appears to affect the methanogen methyl-coenzyme M by inhibiting electron transport during methane production. Thus, the combined impact of nitrate, linseed oil, and anthraquinone on nutrient digestibility and methane emission has been investigated in this study.

Voluntary feed intake is a crucial criterion that has a significant impact on animal productivity and welfare; hence, it is important to check for any response on DMI when evaluating a feed supplement and additives. Feed intake was unaltered in the current experiment, indicating that the diet palatability was not impacted by the methane inhibitors in the control and treated groups of both Sahiwal and Gir breeds. This suggests that the addition of methane inhibitors had no impact on palatability and nutrient intake. Studies have shown inconsistent results regarding the impact of linseed oil and nitrate supplementation on intake and nutritional digestibility. Supplementation of flaxseed oil enhanced the OM and CP digestibility without altering the feed intake. The oil used was roughly 2.4% of the feed intake, which is below the permissible limit for ruminal microbiota activity, which is not high to depress consumption [17]. Kholif et al. [18] also observed that adding flaxseed oil to the diets of goats (20 mL/d) boosted feed utilization and milk production. When linseed oil (2, 3, and 4%) was added to lactating cows' diet, there was no difference in the amount of DM or apparent digestibility of nutrients [19]. It was also observed that nitrate and potassium (2.6 and 4%) in the sheep diet had no impact on the consumption of DM [20,21]. Feeding nitrate has two advantages as it reduces methane emissions while also providing ruminants with non-protein nitrogen. The inclusion of anthraquinone and chloroform during the rearing had no effect on the DMI and growth of dairy calves [22]. In this study, the addition of methane inhibitors had no effect on the apparent digestibility. Supplementation of linseed oil and rubber seed oil could improve nutrient digestibility and rumen fermentation by increasing the vaccenic acid, cis-9 trans-11 conjugated linoleic, and α-linolenic acid composition in dairy lactating cows [23]. The variation could be attributed to species, level of production, and basal diet of the experimental animals.

3.2. Rumen Fermentation Parameters

Rumen fermentation produces excess hydrogen, which must be eliminated from the rumen in order for the fermentation process and microbial development to continue efficiently [24]. Methanogenesis is a crucial mechanism in the rumen for disposing of electrons generated during fermentation, and suppression of methanogens may result in an accumulation of hydrogen that could restrict rumen fermentation. Thus, it is preferable to shift the reducing equivalents from carbon dioxide and hydrogen to processes other

than methanogenesis, such as acetate, and to selectively stimulate fermentation to increase propionate synthesis [25].

There was a significant increase in propionate, ammonia nitrogen, and total nitrogen, and a decrease in butyrate was observed in treated groups of both breeds. This may be due to the shift of H^+ concentrations in the rumen towards propionate and the direct inhibition of methanogens which stimulate acetate production. An increase in H_+ concentrations in the rumen could change feed fermentation pathways to produce a lesser amount of acetate and more propionate, shifting the methanogen community composition away from foremost *Methanobrevibacter* spp. [26]. AQ at 4 ppm increased propionate concentration, which could be a reason for using part of the hydrogen that would be used for microbial lipid production [27]. However, there was no change in acetate and pH was observed between the groups due to the additive effects of methanogenic inhibitors on methanogenesis, or they may be affecting the acetogenic bacterial population [28]. Feed containing plant oils may make the rumen more acidic, lowering the ruminal pH. Ruminal pH and acetate percentage were both decreased ($p < 0.05$) by flaxseed oil in lactating Nubian goats [17]. The impact of nitrate on ruminal fermentation is consistent with the increased rumen pH seen in treated groups after 6 hr of feeding in beef steers [29]. An elevation in ruminal pH might be because the experiments used different types of feed in different studies. Nitrate supplementation enhanced the population of *Prevotella* spp., which may have contributed to the higher propionate proportion [30]. Nitrate supplementation in dairy cows increased dissolved H concentration, microbial N, and propionate molar percentage while reducing ammonia concentrations in rumen fluid and methane emissions [31]. Encapsulated nitrate (ENS) is a feed supplement that reduces *Methanobrevibacter* abundance in the rumen by persistently affecting intestinal methane emission in grazing steers. Furthermore, ENS can stimulate fumarate-reducing and lactate-producing bacteria, lowering acetate synthesis during rumen fermentation [7]. Linseed oil and nitrate addition to ruminant diets also change the VFA profile so that there is more propionate and less acetate [32,33].

3.3. Enteric Methane Emission

In this study, it was observed that supplementation of methane inhibitors to the diet significantly decreased methane emission in the treated groups as compared to control in both breeds approx 20%. Methane reduction may be due to the nitrate and sulfate reduction which is thermodynamically preferred over CO_2 reduction, giving nitrate and sulfate reducing bacteria a competitive edge over methanogens as hydrogen sinks. It was found that feeding Holstein steers with dietary nitrate with six levels (0–3% of feed DM) resulted linear decrease in methane emission [34]. However, supplementation of nitrate and fumarate with a plant leaves-based diet in sheep had no impact on intake/nutrient utilization, blood profile, microbial N, and rumen fermentation parameters [35]. Klop et al. [36] found that the addition of nitrate (1% DM) decreased methane production (g/kg of DMI) by 7.6–10% in dairy lactating cows. It was observed that the addition of linseed oil (4%) with red clover silage-based diet decreased intestinal methane (about 9%), energy losses (about 11%), and N excretion (8%), respectively [37]. Supplementation of linseed oil to ruminant diets had a positive impact on fermentation processes and decreased methane production while increasing propionic acid without interfering with feed digestion [38]. Linseed diet reduced the ruminal acetate proportion, archaea to bacteria ratio, and reduced methane emission in lactating cows [39]. However, under field conditions, a combination of inhibitors may be more useful than an individual due to the synergistic effect of the additive. One product may act on the methanogenic population, the other on its enzyme system, or third act as an H+ sink in the system.

4. Material and Methods

4.1. Experimental Location

The experiment was carried out at the National Dairy Research Institute Livestock Research Center (LRC), Karnal, Haryana, India, which is located at an altitude of 29°42′ N

and 76°54′ E. The minimum and maximum ambient temperature range from near freezing in the winter (4 °C) to 46 °C in the summer, with a diurnal variation of 15–20 °C. The research practice and maintenance were in accordance with the Institute Animal Ethics Committee (IAEC) standards, and consent was obtained from the committee with IAEC approval no. 43-IAEC-18-16.

4.2. Experimental Design

Twelve calves (6–12 months old), six of each breed Sahiwal and Gir, were chosen randomly from the herd at LRC, Karnal, and divided into four groups; Sahiwal control (C) and treated (T) calves; Gir control (C) and treated calves (T) of three calves each based on average body weight. The switch over design was used for periods 1 and 2 (Figure 1). All of the animals were fed in accordance with the ICAR 2013 feeding standard [40]. Animals in all groups were fed chopped oat fodder, wheat straw, and a concentrate mixture. In the control and treated groups, the level of concentrate, oats, and wheat straw were 30:40:30. Additionally, the treated groups were given a meal that included potassium nitrate (1%), linseed oil (0.5%), and anthraquinone (4 ppm). For period 2, as previously stated, the animals were transferred from control to treatment and vice versa.

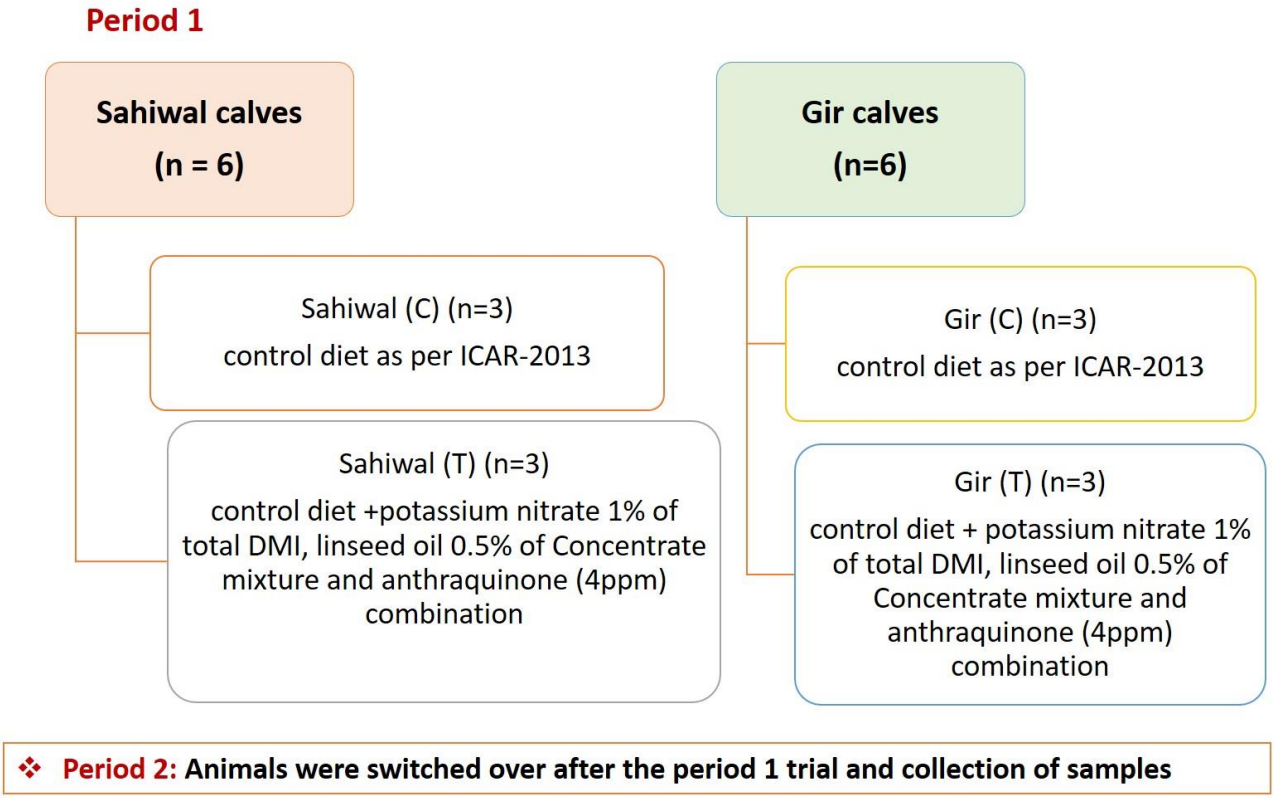

Figure 1. Experimental design for different groups.

4.3. Digestibility Trial

Digestibility trial of seven days collection period was undertaken once in each trial during the last month of the experimental period to assess the nutrient digestibility. The experiment involved housing each animal separately in a shed while making provisions for the quantitative collection of feed/fodders, residues left, and feces. Before beginning the collection, animals were allowed two days to acclimatize in the shed.

4.4. Sampling, Processing, and Storage of Feed Samples

The samples of different feeds offered (concentrate mixture, green fodder, and wheat straw) residues, if any, were taken daily for dry matter (DM) estimation during the

metabolism trial. These samples were pooled at the end of the collection period and ground to pass through a 1 mm sieve and stored in airtight containers. The samples were analyzed for proximate principles (OM, CP, Ash, and EE) and cell wall constituents (NDF and ADF). Feces voided during 24 h were collected daily for seven days and weighed at 9:00 am daily. After thorough mixing, 1/100 of the total sample on a weight basis was kept for DM estimation. Dried pooled fecal samples were ground to pass through a 1 mm sieve size and analyzed for proximate principles and cell wall constituents as per standard procedures. For N estimation of feces, an aliquot of 1/500 of total voided feces were collected daily for seven days and stored in plastic containers containing 25 mL of H_2SO_4 solution.

4.5. Analysis of Feed, Faeces, and Urine Samples

Dried samples of feed offered, residues, and feces were examined for chemical composition, such as dry and organic matter (DM, OM), ethyl ether (EE), crude protein (CP) [41], and cell wall fractions, such as neutral and acid detergent fiber (NDF, ADF) [42].

4.6. Rumen Fermentation Parameters

Rumen liquor was drawn through a stomach tube from various parts of the animal's rumen just before feeding, thoroughly mixed, and strained. The pH of the strained rumen liquor (SRL) was then promptly recorded. Rumen liquor was placed at −20 °C for NH_3-N and total nitrogen estimation. Samples were chilled while being acidified with a few drops of H_2SO_4 (25%) for total and individual volatile fatty acid estimation.

4.6.1. Individual Fatty Acid Estimation (IVFA)

Rumen liquor frozen at 4 °C for 24 h and centrifuged at 3000 rpm. The collected supernatant (4 mL) was treated with meta-phosphoric acid (1 mL) and stored at 4 °C overnight, and centrifuged at 3000 rpm for 10 min to estimate the IVFA via gas chromatography (Nucon 5700, India) fitted with a flame ionization detector (FID) and a stainless-steel column with Chromosorb 101. The injection port, column, and detector were all adjusted at 200, 180, and 210 °C, respectively. The carrier gas (N_2) flow rate through the column was 40 mL/min, whereas the flow rate of H_2 and air via FID were 20 and 300 mL/min, respectively. The sample (3 µL) was injected through the injection port by means of a Hamilton syringe. Diverse VFAs were identified based on the area covered by their retention times on the monitor, and their concentration was determined by comparing the peak areas of the standard [43].

4.6.2. Ammonia and Total Nitrogen Estimation

Rumen liquor (5 mL) and NaOH (5 mL, 40%) were taken in the distillation assembly for the estimation of ammonia. The distillate was collected and titrated against H_2SO_4 (0.01 N) in a conical flask containing boric acid solution (2%, 10 mL). Rumen liquor (2 mL) was placed in a Kjeldahl digestion flask and digested by adding concentrated H_2SO_4 (10 mL). Digested material was diluted with distilled water to a volume of 100 mL. An aliquot of the digested sample (10 mL) was placed in the distillation apparatus, along with NaOH (40%, 15 mL). The distillate was collected in a conical flask with a boric acid solution (2%, 20 mL), and a mixed indicator was taken in the flask and titrated against H_2SO_4 (0.01 N) for the total N estimation [3,4].

4.7. Estimation of Methane Production Using SF6 Method

The enteric methane (CH_4) production was estimated using the SF_6 tracer gas technique for five days. In this method, the permeation tubes were prepared by filling them with a specific amount of SF_6 and inserted into the rumen with a known release rate. Each animal was fitted with a halter and capillary tube attached to an evacuated sampling canister set to fill halfway in 24 h. Sample from the animal's mouth and nose was taken as the vacuum in the sampling canister gradually dissipated. Background CH_4 and SF_6 concentrations were measured for each day by placing one sampling kit in a naturally

ventilated house. The amount of CH_4 and SF_6 in animal samples were then corrected for background concentration. Following sample collection, the canister was pressurized with nitrogen and the concentration of SF_6 was determined using gas chromatography (Nucon 5700), which was equipped with an electron capture detector (250 °C) and a 3.3 m molecular sieve column. To estimate CH_4 concentration, another gas chromatograph was equipped with a flame ionization detector (100 °C) and a stainless-steel column packed with Porapak-Q. In both gas chromatographs, the injector and column were set at 50 and 40 °C, respectively [44].

4.8. Statistical Analysis

One-way analysis of variance was used to analyze the data for nutrient digestibility and methane emission with the help of SPSS software version 9.3. A general linear model was used to differentiate the treatment effect, and the level of significance was considered at 5%. The model used for analysis is $Y_{ij} = \mu + t_i + e_{ij}$, where y_{ij} is the observation on *i*th treatment for *j*th unit, μ is the grand mean, t_i is the effect of *i*th treatment, e_{ij} is random error distributed normally, and *i* is dependent on zero and constant variance. No random effects were considered as all the animals were picked from the herd based on their age and body weight.

5. Conclusions

This study concludes that the addition of methane inhibitors (nitrate, linseed oil, and anthraquinone) to dairy feed can be utilized to lower methane emissions without affecting the digestibility in both Sahiwal and Gir breeds. Combining diverse dietary mitigation strategies could be an effective way to reduce methane emissions while minimizing any negative impacts on calves health and performance to achieve sustainable animal production. However, to attain the optimum results, a thorough study of the rumen microbial population with different concentrations of methane inhibitors and AO's potential health risks is required to scale up at the national level.

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