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Impact of Ground Flaxseed on Methane Reduction and their Influence on Nutrient Digestibility *In-vitro*

Gurjinder Singh[1], Gurpreet Singh[1] and Jaspal Singh Hundal[2]

[1]Dept. of Veterinary Physiology and Biochemistry, [2]Dept. of Animal Nutrition, College of Veterinary Sciences, Guru Angad Dev Veterinary and Animal Sciences University, Ludiana, Punjab (141 004), India

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Corresponding gpsachdeva2000@gmail.com

0000-0003-2235-7476

ABSTRACT

The current study was carried out during the period of October, 2018 to March, 2019 at the Department of Animal Nutrition, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India to assess the effect of the Ground flaxseed supplemented at 0, 5, 10, 15 and 20% of TMR on DM basis on *in-vitro* gas output and fiber degradation. Regardless of the levels of ground flaxseed, the net gas production (NGP) in flaxseed supplemented groups (5–20% level) was lower than control. The partitioning factor (PF) of flaxseed supplemented groups was significantly greater in relation to reference group. The NDF digestibility remained unaffected at supplementation levels ranging from 5% to 15% flaxseed compared to the control. However, it dropped significantly (p<0.01) with flaxseed supplementation at the 20% level. Although, ME availability was not affected by flaxseed supplementation. Significantly (p<0.01) high levels of acetate were observed in control than flaxseed supplemented groups at 15% and 20% levels. Relative proportions of butyrate and fermentable carbon dioxide varied significantly at 5% and 15% levels of flaxseed supplementations. Regardless of the level of flaxseed, methane percent of NGP, methane 100 mg^{-1} DDM and methane 100 mg^{-1} DOM depicted a significant reduction (p<0.01) relative to the control. Ammonia nitrogen levels remained unchanged across all levels of flaxseed supplementation. In conclusion, flaxseed supplementation mitigated the methane production and improved the digestibility without affecting ME availability.

KEYWORDS: Methane estimation, digestibility, volatile fatty acids, flaxseed

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1. INTRODUCTION

Methane (CH_4) being the second-most copious man created greenhouse gas (Myhre et al., 2013), and livestock output is accountable for around a third of human generated CH_4 release. Overall, enteric CH_4 emissions from livestock farms predominates at around 90% (Reisinger et al., 2021), additionally with a share from manure oversight of around 10%, but the need for reduction of this cause is often overlooked (Herrero et al., 2016; Chang et al., 2019). Nevertheless, both US and EU (Leip et al., 2010) have a share of CH_4 release from manure handling of around 25%, showing that manure is a main contributor in some manufacturing systems. Foregut fermenters release notable quantity of CH4, during their digestive processes. During microbial fermentation, they produce CH_4, which is eliminated primarily by the process of eructation and normal breathing and small amounts as flatus (Lassey, 2007). It is estimated that 60–70% enhanced milk and meat will be devoured by 2050 (Makkar, 2016). The emerging future needs for animal source foods are propelled by increase in earnings, inhabitants and industrialization. Animals associated emissions will rise as earth's inhabitants and food requirement increases. The human related emissions of GHGs have risen by 70% from 1970 to 2004 and are supposed to rise further by 25% by 2030 (Rose and McCarl, 2008). The release from livestock is mostly due to emissions from microbial fermentation in foregut fermenters (63%), paddy fields (21%), manure (13%) and burning of crop leftover (2.7%) (Herrero et al., 2009). Since the 1950s, researchers have pursued various strategies to minimize enteric methane emissions. While several methods have demonstrated significant success in reducing emissions and enhancing animal productivity, they are often costly and pose risks to the environment and human health. Therefore, it is essential to evaluate existing techniques and develop improved solutions to mitigate methane emissions from ruminants. Reducing methane emissions from the gut through feeding strategies, additives, or selective breeding requires careful research into the effects on animal welfare and performance. Altering feed composition through dietary manipulation remains the simplest and most cost-effective method to reduce enteric methane emissions (Haque, 2018; Kebreab et al., 2010). Depending on the type and approach of the nutritional intervention, this strategy alone has the potential to reduce ruminant methane emissions by up to 70%. (Benchaar et al., 2001; Mosier et al., 1998). The execution of these measures has progressed slowly (Jayasundara et al., 2016; Melgar et al., 2021). Efforts are ongoing to reduce enteric methane production through dietary adjustments. (Bakshi and Wadhwa, 2009). Recently, increasing government and societal interest in climate change has prompted researchers to focus on understanding rumen methanogenesis and developing strategies to reduce greenhouse gas (GHG) emissions from livestock, particularly enteric methane (CH_4) in ruminants. (Hristov et al., 2013; Congio et al., 2021; Arndt et al., 2022). *In vitro* systems serve as an initial step in evaluating novel nutritional and rumen manipulation strategies. They allow for the rapid screening of a large number of treatments and dosages within a shorter timeframe compared to in vivo experiments. (Flachowsky and Lebzien, 2012; Hristov et al., 2012; Vinyard and Faciola, 2022). Flaxseed is an oilseed which can be used as source of high-quality protein and fat for ruminants (Neveu et al., 2014). Flaxseed contains high levels of linolenic acid, averaging 18% of the total seed weight and 53% of the total fatty acids (Mustafa et al., 2002). Intake of Flaxseed or oil by dairy ruminants has also been shown to mitigate methane production (Chilliard et al., 2009). Added Flaxseed decrease CH_4 emissions because they lower the amount of organic matter that is fermented in the rumen, the activity of the ruminal methanogens, and protozoal numbers (Beauchemin et al., 2009). Besides reducing methane emission, the target of rumen manipulation is to improve fibre degradation, prevent energy losses and improve the health of dairy animals. The systematic study on the effect of flaxseed supplementation on rumen microbes, their activity and rumen fermentation pattern is not available. Given the practicality of in vitro systems for the preliminary evaluation and keeping in view the importance of flaxseed, the present study was planned to evaluate and standardize the dose of ground flaxseed for use in vivo; with the objectives to evaluate the impact of ground flaxseed on *in-vitro* methane reduction and nutrient digestibility.

2. MATERIALS AND METHODS

This research was conducted in the Department of Animal Nutrition at the College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India, from October, 2018 to March, 2019. The investigational protocols involving buffalo calves in this investigation received approval from the Institutional Animal Ethics Committee (IAEC) at Guru Angad Dev Veterinary and Animal Sciences University. Additionally, the study was sanctioned by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) under the Animal Welfare Division of the Ministry of Environment, Forest and Climate Change, Government of India. All experiments with buffalo calves were conducted in compliance with IAEC guidelines.

The present study aimed to evaluate the impact of ground flaxseed on *in-vitro* methane mitigation. Ground flaxseeds were obtained from the local market. A total mixed ration (TMR) was formulated using roughage-to-concentrate ratio of 60% to 40%. The roughage component consisted of wheat straw and berseem forage in an 18:42 proportion. The conventional concentrate mix included 16% deoiled rice bran, 10% soybean meal, 15% wheat, 15% maize, 15% deoiled mustard cake, 10% mustard cake, 15% rice bran, 1% urea, and 2% mineral mixture. The effects of flaxseed, supplemented at 0%, 5%, 10%, 15%, and 20% of the TMR on a dry matter basis, were assessed using the *in-vitro* gas production technique (IVGPT).

2.1. Laboratory-based studies

Male buffaloes with rumen fistulas served as donors for rumen fluid, receiving a diet of 2 kg concentrate blend, 2 kg green fodder with free access to wheat straw. Rumen liquor was collected at 0 hours in Thermos flasks that had been supplied through CO_2 and kept at a temperature of 39°C. The contents of the rumen were blended in a mixer set at 39°C for 2–3 minutes. It is then filtered into and out of two-three layers of cheese cloth. In a 3-liter flask attached to a hotplate mixer within a water heating bath maintained at 39–40°C. Buffer solution was made by combining 0.16 ml of trace-mineral solution, 330 ml of major-mineral solution, 660 ml of bicarbonate buffer, 960 ml of clean water, and 1.6 ml of 0.1% resazurine indicator, with continuous passing of CO_2. The filtered rumen contents were then put to the media in a 1:2 proportion. Approximately 375±5 mg complete feed was weighed in a removable-stem weighing boat and carefully placed at the lowest part of a 100 ml graduated glass syringe to avoid sticking to the walls. Graded levels of ground flaxseed were supplemented at 0%, 5%, 10%, 15%, and 20% on a dry matter basis in the total mixed ration (TMR). A piston, lubricated with Vaseline, was inserted into the container. The syringes, containing sets of three similar samples, were placed in a water bath at 39°C and incubated for 24 hours. If the gas volume in the graduated glass syringe exceeded 75 ml after 7–8 hours, the amount was noted, and the surplus gas was flushed out (Menke et al., 1979; Menke and Steingass, 1988). After about 24–26 hours, the gas generated in every syringe was measured, and the ingredients were conveyed to a beaker and heated with neutral detergent mixture to determine true organic matter and neutral detergent fiber digestibility.

2.2. Methane measurement

To estimate methane production, 200 mg of contents were incubated for a day with rumen fluid and the corresponding different levels of flaxseed in duplicate. Following the incubation time, total gas production was estimated. Gas samples were collected from the topmost part of the glass syringe with the help of an airtight syringe and introduced into a Netchrom 9100 gas chromatograph fitted to a flame ionization detector (FID) and a stainless steel column attached to Porapak-Q. Additionally, methane was determined using an equation derived on the concentrations of volatile fatty acids (VFA) (Wolin, 1960).

2.3. Determination of volatile fatty acids (VFAs)

Estimation of Volatile Fatty Acids was done as per the standard procedure explained by (Cottyn and Boucque, 1968).

2.4. Analytical methods

The finely crushed mixture samples were examined for proximate analysis and ammonia measurement. Neutral detergent fiber (NDF) was measured as per the methods of Robertson and Van Soest (1991). For ammonia estimation, 5 ml of the supernatant was mixed with 1 N NaOH and subjected to steam distillation. The released NH_3 was captured in a boric acid solution containing a mixed indicator and then titrated with 0.01 N H_2SO_4.

2.5. Statistical evaluation

The gathered data were analyzed following the methods outlined by Snedecor and Cochran (1994). Significant distinctions between means were assessed employing Tukey's b test.

3. RESULTS AND DISCUSSION

Flaxseed supplementation at 20% improved NDF digestibility compared to the control. It may be due to enhanced activity of microflora (Table 1). However, digestibility of TOM was not significantly affected. Irrespective of the levels of flaxseed, The Partitioning Factor (PF), which indicates the substrate-dependent variation in the ratio of substrate degradation to gas volume produced during different incubations, was significantly (p<0.01) higher when compared to control showing higher digestibility. There was no significant difference as far as ME and ammonia nitrogen was concerned in any of the groups with slight decrease in NGP irrespective of the levels of supplementation of ground flaxseed.

Regardless of the flaxseed level, the production of total volatile fatty acids (TVFAs), acetate, and propionate (Table 2) was not altered however production of acetate was significantly raised at 15 and 20% levels with no change in butyrate. The reason for rise in acetate could be the dietary fibre (DF) content in the flaxseed that lead to enhanced production of acetate following supplementation of flaxseed. Flaxseed dietary fiber (DF) has been noted for its various beneficial effects, including enhancing perceived satiety. Ibrugger et al. (2012) enhancing fat excretion Kristensen et al. (2012), improving constipation Cunnane et al. (1995) and It also has a particular impact on intestinal microbiota, stimulating the production of short-chain fatty acids (SCFAs) that influence host metabolism. Arora et al. (2019). Therefore, flaxseed dietary fiber (DF) is an

Table 1: Effect of graded levels of ground flaxseed on *in vitro* net gas production, digestibility of nutrients using wheat straw as substrate/fermentation parameters of TMR at 24h

Parameters	T_1 (0%)	T_2 (5%)	T_3 (10%)	T_4 (15%)	T_5 (20%)	PSE	*p* value
NGP, ml g^{-1}	146.21	145.3	143.37	141.75	140.87	2.978	0.682
NDFD*	47.74^{a}	48.58^{ab}	47.76^{a}	49.30^{ab}	50.30^{b}	0.187	0.012
TOMD	69.39	69.45	69.24	70.29	69.96	0.139	0.226
PF, mg ml^{-1**}	2.47^{a}	2.57^{b}	2.62^{bc}	2.65^{c}	2.64^{c}	0.006	0.001
ME, MJ kg^{-1}	7.29	7.33	7.20	7.11	7.13	0.039	0.392
Ammonia-N, mg dl^{-1}	0.015	0.016	0.016	0.017	0.017	0.000	0.109

NGP: Net gas production; D: Digestibility; NDF: Neutral detergent fiber; TOM: True organic matter; TDDM: True digestible dry matter; PF: Partioning factor; ME: Metabolizable energy; Figures with different superscripts in a row differ significantly, $^{**}p<0.01$, $^{*}p<0.05$

Table 2: Effect of graded levels of ground flaxseed on the *in vitro* volatile fatty acid production (mM dl^{-1})

Parameters	T_1 (0%)	T_2 (5%)	T_3 (10%)	T_4 (15%)	T_5 (20%)	PSE	*p* value
TVFA	5.04	5.25	5.26	5.23	5.25	0.030	0.242
Acetate*	3.19^{a}	3.28^{ab}	3.34^{ab}	3.38^{b}	3.40^{b}	0.014	0.022
Propionate	1.18	1.23	1.17	1.17	1.18	0.019	0.819
Isobutyrate	0.0418	0.0316	0.0393	0.0387	0.0340	0.001	0.125
Butyrate	0.429	0.492	0.468	0.415	0.444	0.008	0.122
Isovalerate	0.198	0.211	0.195	0.214	0.189	0.005	0.468
A:P	2.69	2.66	2.88	2.89	2.87	0.057	0.587

Figures with different superscripts in a row differ significantly, $^{*}p<0.01$

excellent source of supplemental fiber, offering a range of benefits for animals. However, acetate to propionate ratio did not show any significant variations. Whereas, Resende et al. (2015) observed that the ruminal molar proportion of propionate increased linearly, following supplementation of incremental levels ground flaxseed in dairy cows fed high-forage diets. The relative proportion of butyrate (Table 3) was not significantly altered in different groups when compared to control. However there was ($p<0.01$) reduction in butyrate proportions in treatment groups T_2 and T_4 supplemented with flaxseed at 5% and 15% levels respectively. In contrast, Huang et al. (2021) observed that compared to the control check group (CK), the molar proportion of acetate increased in the Whole flaxseed (WF) and ground flaxseed (GF) groups and was highest in the ground flaxseed group ($p<0.001$). In addition, propionate, isobutyrate, butyrate, isovalerate, and valerate showed the same trend CK<WF<GF ($p<0.05$). Ground and WF supplementation could decrease the ratio of acetate to propionate ($p<0.001$). Fermentable carbon dioxide also

Table 3: Effect of graded levels of ground flaxseed on relative proportion of different volatile fatty acids

Parameters	T_1 (0%)	T_2 (5%)	T_3 (10%)	T_4 (15%)	T_5 (20%)	PSE	*p* value
Acetate	63.27	62.48	63.63	64.77	64.74	0.543	0.650
Propionate	23.47	23.51	22.19	22.44	22.55	0.257	0.436
Isobutyrate	0.829	0.602	0.749	0.740	0.648	0.022	0.103
Butyrate*	8.508^{ab}	9.37^{b}	8.877^{ab}	7.947^{a}	8.445^{ab}	0.105	0.045
Isovalerate	3.919	4.026	3.706	4.102	3.612	0.076	0.324

Figures with different superscripts in a row differ significantly, $^{*}p<0.01$

Table 4: Fermentation parameters and hydrogen balance of different TMRs

Parameters	T_1 (0%)	T_2 (5%)	T_3 (10%)	T_4 (15%)	T_5 (20%)	PSE	*p* value
FCO_2, mmol*	50.27^{ab}	51.18^{b}	50.68^{ab}	49.91^{a}	50.67^{ab}	0.096	0.045
FCH_4; mmol	30.02	30.05	30.70	30.74	30.95	0.289	0.776
HR, %	92.52	90.3	90.0	90.28	90.0	0.261	0.109
HC via CH_4/VFA	1.65	1.55	1.68	1.72	1.69	0.043	0.743
FE, %	75.42	75.78	74.98	74.86	74.93	0.156	0.551
VFA UI	3.42	3.45	3.68	3.60	3.62	0.058	0.610

F: Fermentative; H: Hydrogen; R: Recovery; C: Consumed; E: Efficiency; I: Index; CO_2: Carbondioxide; CH_4: methane; VFA: Volatile fatty acids; U: Utilization; Figures with different superscripts in a row differ significantly; $^*p<0.05$

followed the similar pattern (Table 4). Rest of the parameters did not show any significant alterations in different groups.

Methane 100 mg^{-1} DDM and DOM was significantly reduced following supplementation regardless of the levels of ground flaxseed. Flaxseed supplemented groups at all levels have significantly reduced methane emission shown by significantly reduced ($p<0.01$) methane percent of NGP (Table 5). Similarly, Chilliard et al. (2009) reported that the consumption of flaxseed or flaxseed oil by dairy ruminants has been found to reduce their methane production. In another study, Almeida et al. (2023) also noted that enteric CH_4 production tended to decrease linearly ($p=0.055$) with feeding incremental amounts of ground flaxseed in lactating dairy cows. The reduction in methane may be due to reduction in population of methanogenic bacteria. Li et al. (2012) also reported reduction in archaeal abundance, their gene expression and the activity of enzymes that contribute to methane production in the rumen of dairy cows following supplementation of flaxseed.

Table 5: Effect of graded levels of ground flaxseed on *in vitro* methane production

Parameters	T_1 (0%)	T_2 (5%)	T_3 (10%)	T_4 (15%)	T_5 (20%)	PSE	*p* value
CH_4, % of NGP***	10.1^{d}	9.08^{c}	8.07^{b}	7.46^{ab}	7.29^{a}	0.080	0.000
CH_4, ml 100 mg^{-1} DDM***	6.59^{d}	5.94^{c}	5.27^{b}	4.75^{a}	4.63^{a}	0.056	0.000
CH_4, ml 100 mg^{-1} DOM***	4.87^{d}	4.31^{c}	3.78^{b}	3.42^{ab}	3.33^{a}	0.043	0.000

DDM: Digestible dry matter; DOM: Digestible organic matter; Figures with different superscripts in a row differ significantly, $^{***}p<0.001$

4. CONCLUSION

Based on the *in-vitro* observations of net gas production (NGP), methane emissions, volatile fatty acid (VFA) profiles, nutrient digestibility, and metabolizable energy (ME) availability from the substrate, a 15% inclusion level of ground flaxseed on a dry matter basis in the total mixed ration (TMR) was determined to be the optimal level for subsequent in-vivo studies.

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