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Effect of lucerne straw based total mixed rations on *in vitro* digestibility and methane emission

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Abstract
This study set out to assess the impact of a Total Mixed Ration (TMR) based on lucerne straw on methane production and *in vitro* digestibility. Different TMRs were created using concentrates and wheat straw in a 30:70 ratio, which served as the substrate for *in vitro* study. As a control, the TMR without lucerne straw was assigned the label L_0. The study examined the *in vitro* DMD and methane production of TMRs that replaced 10%, 20%, 30%, 40%, and 50% of the wheat straw with lucerne straw. These TMRs were designed as L_1, L_2, L_3, L_4, L_5, and L_6, respectively. Gas chromatography was used to estimate the production of methane. When lucerne straw was included in TMRs at all levels, *in vitro* experiments showed an improvement in dry matter digestibility when compared to the control group. However, adding 35% lucerne straw to TMR instead of 35% wheat straw resulted in a significant (p<0.05) increase in *in vitro* DM digestibility (9.70%). Similar to the control group, all lucerne straw-based TMRs showed a decrease in *in vitro* methane generation. Compared to the control group, which produced 2.34 ml/100 mg DM, *in vitro* methane production was 20.94% lower in TMR containing 35% lucerne straw (1.85 ml/100 mg DM). Compared to the control group, *in vitro* methane production dropped by 27.76% in terms of ml/100 mg DMD at a 35% inclusion level of lucerne straw. Therefore, adding 50% (w/w) more lucerne straw to 70% of the wheat straw in a 70:30 TMR blend increases DMD and decreases methane synthesis *in vitro*.

Keywords: *IVDMD, In vitro* gas production technique, *in vitro* methane production, lucerne straw

Introduction
Global warming is the increase in global atmospheric temperature due to increase in Green-house gases mainly carbon dioxide (CO_2), methane (CH_4) and nitrus oxide (N_2O). Livestock's enteric fermentation is a significant contributor of methane, with a global warming potential 28 times higher than that of carbon dioxide [6]. Enteric methane contributes ~ 44% among GHGs emitted from the livestock sector [4]. As per the report of Indian Network on Climate Change Assessment [5], the ruminants contribute up to 50% of the total methane emission, of which enteric fermentation contributes > 49% in India. In addition to its connection with environmental issues, it also signifies a specific level of energy depletion in animals [3]. *In vitro* studies have revealed that legume straw produce less methane than cereal straws [1]. Hence efforts were made to study the effect of inclusion of lucerne straw in TMR replacing wheat straw on *in vitro* methane emission.

Materials and Methods
Preparation of Diets
In vitro experiment was conducted using total mixed rations (TMRs) prepared by mixing concentrates and wheat straw in a 30:70 ratio. The control group (L0) has been designated as the TMR without lucerne straw. The study investigated the *in vitro* dry matter digestibility (DMD) and the production of methane of total mixed rations (TMRs) containing varying levels of lucerne straw (10%, 20%, 30%, 35%, 40%, and 50%) replacing wheat straw. These levels were designated as L_1, L_2, L_3, L_4, L_5, and L_6, respectively. The table presents the component composition of TMR with varying quantities of lucerne straw given in Table 1.

Table 1: Ingredient composition of TMR with different levels of lucerne straw

Ingredients	Total mixed ration with different levels of lucerne straw						
	L_0	L_1	L_2	L_3	L_4	L_5	L_6
Wheat straw	70	60	50	40	35	30	20
Lucerne straw	0	10	20	30	35	40	50
Maize	5	5	10	13	10	18	19
Soyabean meal	10	8	5	2	0	0	0
De-oiled rice bran	5	7	5	5	9	1	0
Molasses	9	9	9	9	10	10	10
Mineral mixture	1	1	1	1	1	1	1

400 ml of rumen fluid was obtained from two mature crossbred bullocks. A maintenance ration was given to the bullocks, and collecting took place two hours following feeding. Using a stomach tube and a suction pump under negative pressure, the rumen liquor was collected. By employing a four-layer muslin cloth to filter the rumen liquor, a substance known as Strained Rumen Liquor (SRL) was produced. The SRL was transported to the laboratory in a preheated thermos flask with a reading of 39±1°C. Following its exposure to carbon dioxide gas, the SRL was preserved at a consistent temperature of 39±1°C in preparation for the subsequent analysis. The feed samples were pulverized using a 10 mm sieve. For the purpose of estimating methane, an approximate volume of 200 mg of TMR samples was weighed into 100 ml calibrated *in vitro* glass vials. Conversely, in order to evaluate digestibility, 500 mg of TMR samples were weighed. A mineral solution was prepared and subsequently agitated in a water bath set at 39°C with CO2 being flushed continuously [9]. Three vials were maintained devoid of any contents. The vials were agitated while being stored in a water bath that was adjusted to a temperature of 39°C. A flask containing the specified quantities of water, micro and macro solutions, buffer, and resazurin was transferred to an incubator preheated to 39°C. A predetermined volume of rumen fluid was added to the vessel containing the medium, while CO_2 was introduced into the medium in a continuous stream. For methane estimation, 30 ml of buffered rumen fluid was dispensed into syringes containing the TMR samples, whereas for DMD estimation, 40 ml was dispensed. Every stage of the procedure was consistently purged with carbon dioxide.

Determination of *in vitro* digestibility

The objective of the *in vitro* investigations was to ascertain the *in vitro* dry matter digestibility (DMD) using 500 mg of TMR as the substrate, supplemented with varying concentrations of lucerne straw. The *in vitro* digestibility was assessed following a 24-hour incubation period. Each syringe's contents were dried, weighed, and then filtered through a Gooch crucible that had been pre-weighed. After being oven dried for 24 hours at 70°C, the Gooch crucibles containing the undigested remains were cooled in desiccators and the dry matter was measured by weight and *in vitro*. DMD was calculated.

Determination of *In vitro* Methane Production

The *in vitro* methane production was determined by measuring the gas produced in 100 ml glass syringes after incubating 200 mg of substrate for 24 hours. A volume of 1 ml of gas was injected into the Gas Chromatograph from each syringe, and the concentrations of methane were calculated by comparing them to a standard methane gas. The samples were tested three times using a gas chromatography (GC) apparatus. The instrument was equipped with a stainless steel column that was 4 feet long and had an inner diameter of 3.2 mm. The column was packed with Porapack N (80 to 100 mesh) and had a flame ionization detector (FID) attached.

A column temperature of 50°C was maintained, and nitrogen was employed as the carrier gas at a rate of 30 ml/min. The standards (102 ppm) utilised for calibration were obtained from chemix specialty gases and equipment, Bangalore.

Results and Discussion

***In vitro* Dry Matter Digestibility**

The results of *in vitro* DM digestibility (DMD) of TMR with or without lucerne straw are presented in Table 2. *In vitro*, DMD increased in all lucerne straw-supplemented TMRs compared to TMRs without lucerne straw. The L4 (72.61%), L5 (74.21%), and L6 (77.52%) groups had considerably higher *in vitro* DMD ($p<0.05$) than the control (L0) group (66.19%), while other groups had lower *in vitro* DMD.

These findings are in line with results reported by earlier observations where *in vitro* digestibility improved by 5.25% due to supplementation of red gram straw and by 1.43% due to supplementation of black gram straw as compared to maize stover[7]. Similarly, 10.43% higher *in vitro* DMD reported in legume straw based TMR, as compared to wheat straw based TMR [11]. Similar to our finding, 40.31% higher *in vitro* DMD observed in pigeon pea straw based TMR, as compared to wheat straw based TMR [2]. The higher digestibility in legume straws is on account of better nitrogen and energy content compared to cereal straw.

Table 2: *In vitro* Dry matter digestibility & Methane production of TMRs with different levels of lucerne straw

Groups	DMD (%)	CH_4 ml/100mg DM	CH_4 ml/100mg DDM
L_0 (0% lucerne straw)	66.19^{c}±1.31	2.34±0.21	3.53±0.31
L_1 (10% lucerne straw)	71.69^{bc}±2.38	2.17±0.21	3.08±0.29
L_2 (20% lucerne straw)	73.27^{ab}±2.57	2.30±0.39	3.16±0.53
L_3 (30% lucerne straw)	68.91^{bc}±0.56	1.47±0.18	2.14±0.27
L_4 (35% lucerne straw)	72.61^{ab}±1.75	1.85±0.22	2.55±0.31
L_5 (40% lucerne straw)	74.21^{ab}±1.65	1.84±0.07	2.68±0.51
L_6 (50% lucerne straw)	77.52^{a}±1.72	1.93±0.10	2.72±0.36

ABC Means with different superscripts in columns for a parameter differ significantly ($p<0.05$)

In vitro Methane production in terms of ml/100 mg DM

In vitro methane production decreased in all lucerne straw supplemented TMRs, as compared to TMR without lucerne straw. The lowest *in vitro* methane production (ml/100 mg DM) was found in L_3 (1.47), L_4 (1.85) and L_5 (1.84) groups which was 37.18%, 20.94% & 21.37% lower than the control (L_0) group (2.34), respectively.

Methane emission is inversely related to digestibility of feed. Also it is well established that plant metabolites like tannins and saponins decrease methane emission in ruminants. In present study, the higher digestibility and presence of saponins in lucerne straws might be the reason for decrease in methane emission. In other studies also, *in vitro* CH_4 Production has been found lower in legume straw as compared to cereal Straw based TMR [1, 11]. Similarly, *in vitro* methane production **r**educed by 9.69% in pigeon pea straw based TMR as compared to wheat straw based TMR[2]. Ssupplementation of lucerne fodder at 15, 30 and 45% to wheat straw and concentrate based diet significantly reduced *in vitro* methane production by 8, 15 and 18%, respectively [8]. Similar to our finding, 9.72% reduction in methane production reported in legume straw based TMR group compared to the value observed in wheat straw based TMR group [11].

In vitro Methane production in terms of ml/100 mg DMD

The *in vitro* methane production (ml/100 mg DMD) was also lower in all lucerne straw supplemented TMRs compared to control group. The lowest *in vitro* methane production (ml/100 mg DDM) was found in L_3 (2.14), L_4 (2.55) and L_5 (2.68) groups which was 39.38%, 27.76% & 24.08% lower than the control (L_0) group (3.53) respectively. Similar to present findings, *in vitro* methane production reduced by 18.16% in terms of ml/l00 mg DMD in legume straw based TMR, as compared to wheat straw based TMR [11]. Similarly, *in vitro* methane production significantly reduced by 18.16% in pigeon pea straw based TMR, as compared to wheat straw based TMR [2].

Conclusion

Present study revealed that replacement of cereal straw by lucerne straw @50% (w/w) in TMR has potential for improving dry matter digestibility and reducing methane production.

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