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DOSE-DEPENDENT EFFECTS OF POLYHERBAL MIXTURE SUPPLEMENTATION ON METHANE MITIGATION, FERMENTATION CHARACTERISTICS, AND IN VITRO DRY MATTER DIGESTIBILITY USING THE GAS PRODUCTION TECHNIQUE

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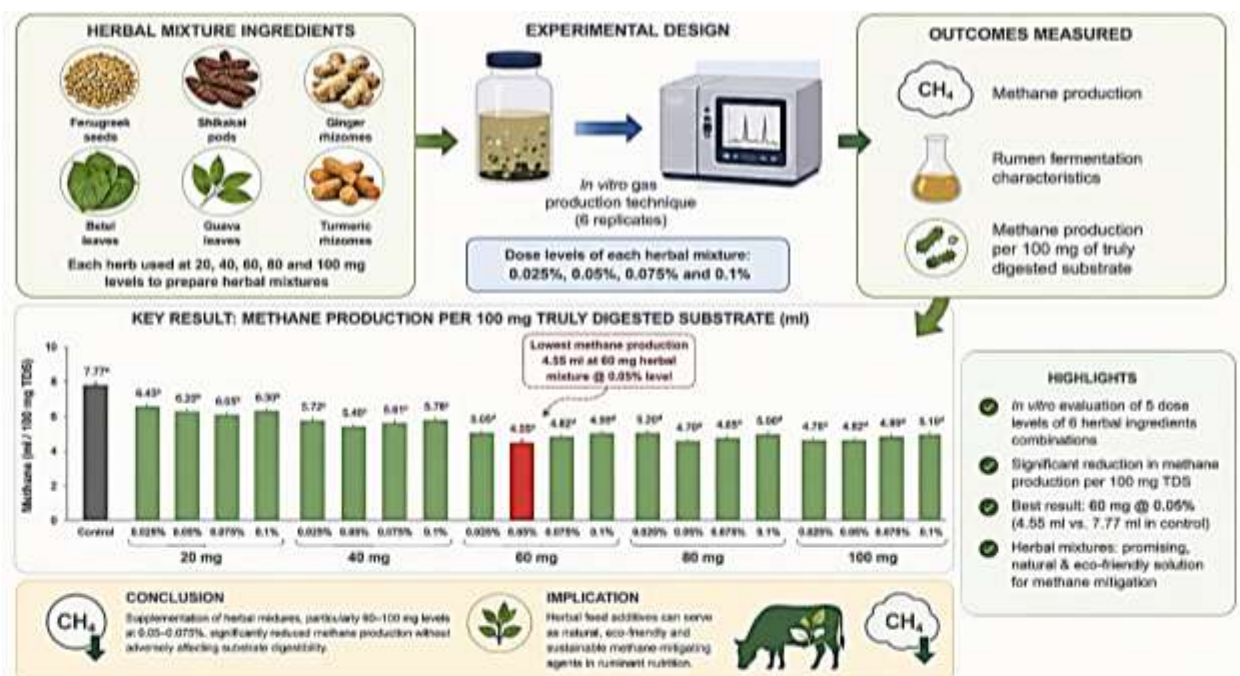
ABSTRACT

An in vitro study was conducted to evaluate the methane mitigation potential of selected herbal mixtures containing fenugreek seeds, shikakai pods, ginger rhizomes, betel leaves, guava leaves, and turmeric rhizomes. Different herbal mixture combinations were prepared by incorporating each herb at 20, 40, 60, 80, and 100 mg levels. Each herbal combination was further evaluated at four dose levels, viz., 0.025%, 0.05%, 0.075%, and 0.1%, using the in vitro gas production technique in six replicates. Methane production, rumen fermentation characteristics, and methane production per 100 mg of truly digested substrate were assessed to determine the efficacy of the herbal mixtures in reducing enteric methane emission. The results revealed that methane production per 100 mg of truly digested substrate was significantly ($P<0.05$) reduced in several herbal mixture combinations compared to the control group (7.77 ml). Among the treatments, the 60 mg herbal mixture at 0.05% supplementation level exhibited the lowest methane production (4.55 ml), followed by 80 mg at 0.05% (4.70 ml), 100 mg at 0.05% (4.76 ml), and 100 mg at 0.075% (4.82 ml). The findings indicate that herbal feed additives have considerable potential as natural, eco-friendly methane-mitigating agents in ruminant nutrition without adversely affecting substrate digestibility.

KEYWORDS: Methane mitigation, herbal mixtures, in vitro fermentation, ruminants, rumen fermentation, phytogenic feed additives, methane production.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Ruminants possess a unique ability to convert fibrous lignocellulosic agro-industrial by-products into valuable products such as milk and meat through microbial fermentation in the rumen. The rumen ecosystem consists of a diverse population of microorganisms, including bacteria, protozoa, fungi, and methanogenic archaea, which play a crucial role in the digestion of complex carbohydrates and the production of volatile fatty acids (VFAs) such as acetate, propionate, and butyrate, along with gases including carbon dioxide and methane (McSweeney and Mackie, 2012). Methane production during enteric fermentation represents a significant loss of dietary energy and contributes substantially to greenhouse gas emissions from the livestock sector. Methane is considered the second most important greenhouse gas after carbon dioxide due to its high global warming potential. It possesses nearly 23 times greater warming potential than carbon dioxide over 100 years (IPCC, 1996). Increased atmospheric methane concentration has been associated with global climate change, rising temperatures, and environmental degradation. The Intergovernmental Panel on Climate Change (IPCC, 2001) projected that the global average temperature may increase by 2–4.5 °C by the end of the century, with an estimated warming rate of approximately 0.2 °C per decade. In tropical regions, where ruminants are predominantly fed low-quality fibrous diets, methane emission may account for approximately 10–12% of gross dietary energy intake, thereby reducing feed efficiency and animal productivity (McCrabb and Hunter, 1999).

Globally, the livestock sector contributes significantly to anthropogenic methane emissions, accounting for nearly 14.5% of total greenhouse gas emissions (Gerber et al., 2013). Consequently, considerable research attention has been directed toward developing effective and sustainable methane mitigation strategies in ruminants. Various approaches, including feed processing, dietary manipulation, defaunation, supplementation of organic acids, ionophores, probiotics, prebiotics, surfactants, halogenated methane analogues, sulphates, and unsaturated fatty acids, have been investigated for their potential to reduce enteric methane production. However, several of these methods are associated with limitations such as high cost, microbial resistance, reduced feed intake, and possible residual effects in animal products. n recent years, phytogenic feed additives and herbal supplements have emerged as promising natural alternatives for methane mitigation in ruminants. Herbal plants such as neem (*Azadirachta indica*), garlic (*Allium sativum*), ginger (*Zingiber officinale*), kadukka (*Terminalia chebula*), fenugreek (*Trigonella foenum-graecum*), guava (*Psidium guajava*), betel (*Piper betle*), and turmeric (*Curcuma longa*) contain a variety of bioactive secondary metabolites, including tannins, saponins, flavonoids, terpenoids, alkaloids, phenolic compounds, and essential oils. These phytochemicals possess antimicrobial properties capable of selectively modulating rumen microbial populations, particularly methanogenic archaea and protozoa, thereby reducing methanogenesis without adversely affecting rumen fermentation and nutrient digestibility (Patra and Saxena, 2010; Bunglavan, 2014). Herbal feed additives are considered environmentally safe, biodegradable, and free from harmful chemical residues, making them suitable alternatives to synthetic feed additives. Furthermore, these herbal products have been traditionally used for centuries in human and animal health applications with minimal adverse effects. Therefore, the present study was undertaken to evaluate the methane mitigation potential of selected herbal mixture combinations at different supplementation levels using an in vitro gas production technique.

2. MATERIALS AND METHODS

2.1 Preparation of Herbal Mixtures

Five herbal feed additives, namely shikakai pods (*Acacia concinna*), ginger rhizomes (*Zingiber officinale*), betel leaves (*Piper beetle*), guava leaves (*Psidium guajava*), and turmeric rhizomes (*Curcuma longa*), were selected for the present study based on their reported antimicrobial and methane-mitigating properties. The herbal materials were collected, shade-dried, finely powdered, and used for the preparation of different herbal mixture combinations. Separate herbal mixture combinations were prepared by incorporating each herb at levels of 20, 40, 60, 80, and 100 mg, respectively. From each herbal mixture combination, four supplementation levels, viz., 0.025%, 0.05%, 0.075%, and 0.1%, were evaluated for their methane mitigation potential under in vitro conditions. Each treatment was carried out in six replicates along with a control group without herbal supplementation. The figure 1 illustrates the overall methodology adopted for evaluating the methane mitigation potential of selected herbal feed additives under in vitro rumen fermentation conditions. Initially, different herbal materials including shikakai pods (*Acacia concinna*), ginger rhizomes (*Zingiber officinale*), betel leaves (*Piper beetle*), guava leaves (*Psidium guajava*), and turmeric rhizomes (*Curcuma longa*) were selected as feed additives. The collected plant materials were shade-dried, finely ground, and converted into powder form. Herbal mixtures were then prepared at different inclusion levels (20, 40, 60, 80, and 100 mg). Subsequently, supplementation levels of 0.025%, 0.05%, 0.075%, and 0.1% were tested for each herbal mixture. The experimental setup consisted of six replicates for each treatment along with a control group without herbal supplementation. Finally, the methane mitigation potential of the herbal mixtures was evaluated using the in vitro gas production technique. Figure 1 presents the Schematic Representation of Herbal Mixture Preparation and Experimental Design for In Vitro Methane Mitigation Study.

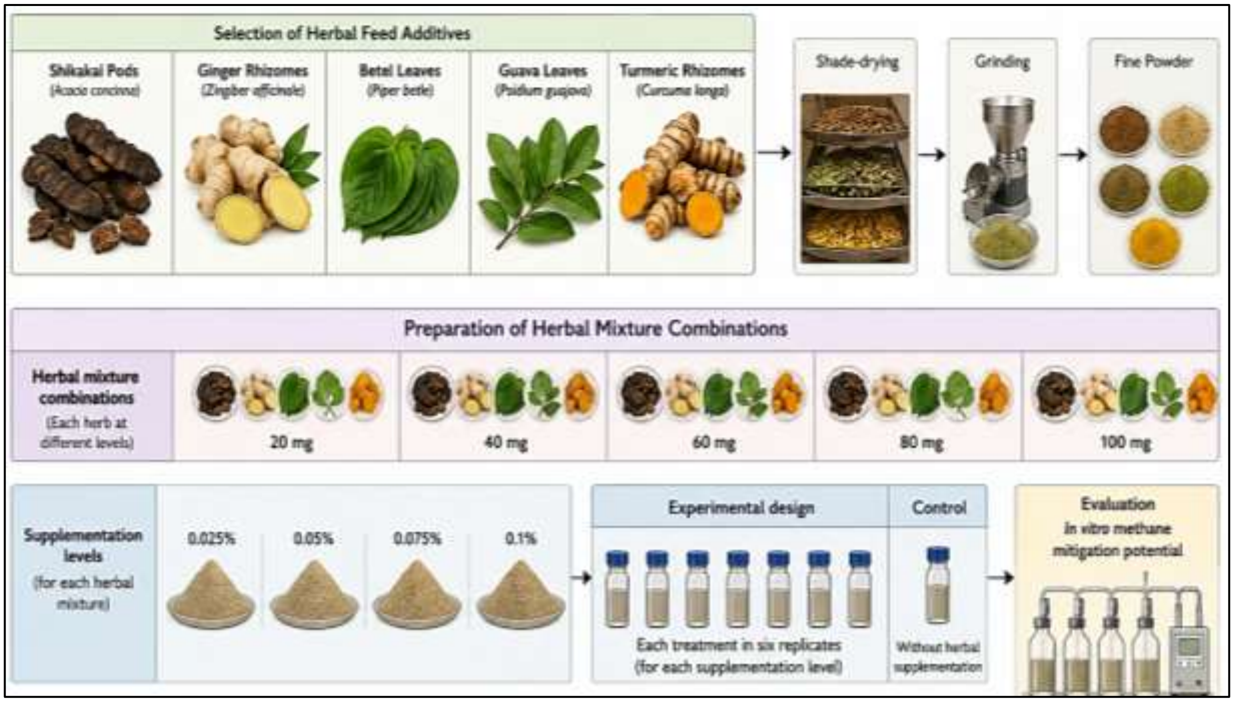


Figure 1: Schematic Representation of Herbal Mixture Preparation and Experimental Design for In Vitro Methane Mitigation Study

2.2 In Vitro Gas Production Study

The in vitro gas production study was conducted using the Hohenheim gas production technique described by Menke and Steingass (1988). Approximately 200 mg of experimental substrate was weighed into calibrated glass syringes. Different herbal mixture combinations prepared from fenugreek seeds, shikakai pods, ginger rhizomes, betel leaves, guava leaves, and turmeric rhizomes were added at supplementation levels of 0.025%, 0.05%, 0.075%, and 0.1% of substrate dry matter. Buffered rumen liquor was prepared by mixing rumen fluid collected from fistulated cattle with artificial saliva buffer under continuous flushing of CO_2 to maintain anaerobic conditions. The syringes containing substrate and herbal mixtures were inoculated with buffered rumen liquor and incubated at 39°C for 24 h. Each treatment was maintained in six replicates along with a control group without herbal supplementation. Total gas production was recorded from the calibrated syringes after incubation to assess rumen fermentation characteristics and methane mitigation potential of the herbal mixtures. The experimental approach was designed based on previous reports indicating that herbal extracts rich in tannins, saponins, flavonoids, and essential oils can influence rumen fermentation and reduce methane production.

2.3 Estimation of Methane Production

Methane concentration and methane emission from the control and herbal mixture supplemented groups were estimated according to the method described by Sitaula et al. (1992). Gas samples collected from the headspace of incubated syringes after completion of fermentation were transferred into airtight gas sampling vials for methane analysis. Methane estimation was carried out using gas chromatography (Perkin Elmer, Clarus 500 model) fitted with a Flame Ionization Detector (FID) and a capillary column of 30 m length and 250 µm internal diameter. Nitrogen was used as the carrier gas. The injector, detector, and oven temperatures were maintained according to standard operating conditions for methane determination. Methane concentration was expressed as percentage methane in total gas production and calculated as methane output per gram of dry matter incubated.The methane mitigation potential of the herbal mixtures was evaluated by comparing methane production in the supplemented groups with the control group. Herbs such as shikakai, ginger, betel leaves, guava leaves, and turmeric rhizomes were selected based on previous findings demonstrating their antimethanogenic activity due to the presence of plant secondary metabolites such as tannins, saponins, flavonoids, and essential oils.

2.4 In Vitro True Dry Matter Digestibility (IVTDMD)

The in vitro true dry matter digestibility (IVTDMD) of fermented feed samples was determined following the method of Van Soest and Robertson (1988). After completion of the in vitro incubation period, the contents of each syringe were filtered and the undigested residues were subjected to neutral detergent solution (NDS) treatment to remove microbial debris and soluble components. The residues obtained after NDS treatment were dried in a hot air oven at

105°C until constant weight was attained. The IVTDMD was calculated as the difference between the initial dry weight of the incubated substrate and the dry weight of the residue remaining after neutral detergent extraction. Digestibility values were expressed as percentage dry matter digestibility. The IVTDMD values obtained for the herbal mixture supplemented groups were compared with the control group to evaluate the effect of herbal supplementation on nutrient utilization and rumen fermentation efficiency. Previous studies reported that certain herbal extracts such as shikakai pods, betel leaves, guava leaves, and turmeric rhizomes may influence dry matter digestibility due to the presence of tannins and other bioactive compounds.

2.5 Calculation of Methane Production per 100 mg Truly Digested Substrate

Methane production per 100 mg of truly digested substrate was calculated to evaluate the efficiency of methane emission relative to the actual degraded substrate during in vitro fermentation. The methane output was expressed using the following formula:

Methane production per 100 mg truly digested substrate= Methane emission (ml) ×0.1/ Degradability

Where methane emission was recorded in milliliters, and degradability represented the amount of substrate truly digested during the incubation period. This calculation enabled comparison of methane production efficiency among different herbal mixture supplementation levels by relating methane output to substrate utilization.

2.6 Statistical Analysis

The experimental data obtained from the in vitro gas production study, methane estimation, and in vitro true dry matter digestibility (IVTDMD) analyses were subjected to statistical analysis using one-way analysis of variance (ANOVA) with IBM SPSS Statistics software version 20.0 for Windows, following the procedures described by Snedecor and Cochran (1994). All observations were expressed as mean ± standard error (SE). The significance of differences among treatment means was determined using Duncan's Multiple Range Test (DMRT). Differences among the treatment groups were considered statistically significant at $P < 0.05$. The statistical analysis was carried out to evaluate the effect of different herbal mixture combinations and supplementation levels on rumen fermentation characteristics, methane production, and digestibility parameters. Figure 2 illustrates the methodology used for calculating methane production per 100 mg of truly digested substrate and the statistical analysis employed in the in vitro rumen fermentation study. The upper section presents the workflow involved in methane estimation, including in vitro incubation using the Hohenheim gas production technique, total gas collection, gas sampling, and methane analysis by gas chromatography. The formula used for methane production calculation based on methane emission and degradability is also depicted. The lower section summarizes the statistical analysis procedure followed for evaluating experimental data. The workflow includes data collection, data entry into IBM SPSS Statistics software (version 20.0), one-way ANOVA, Duncan's Multiple Range Test (DMRT), and presentation of results as mean ± standard error (SE). Representative graphs showing methane production and IVTDMD values among treatments are also included.

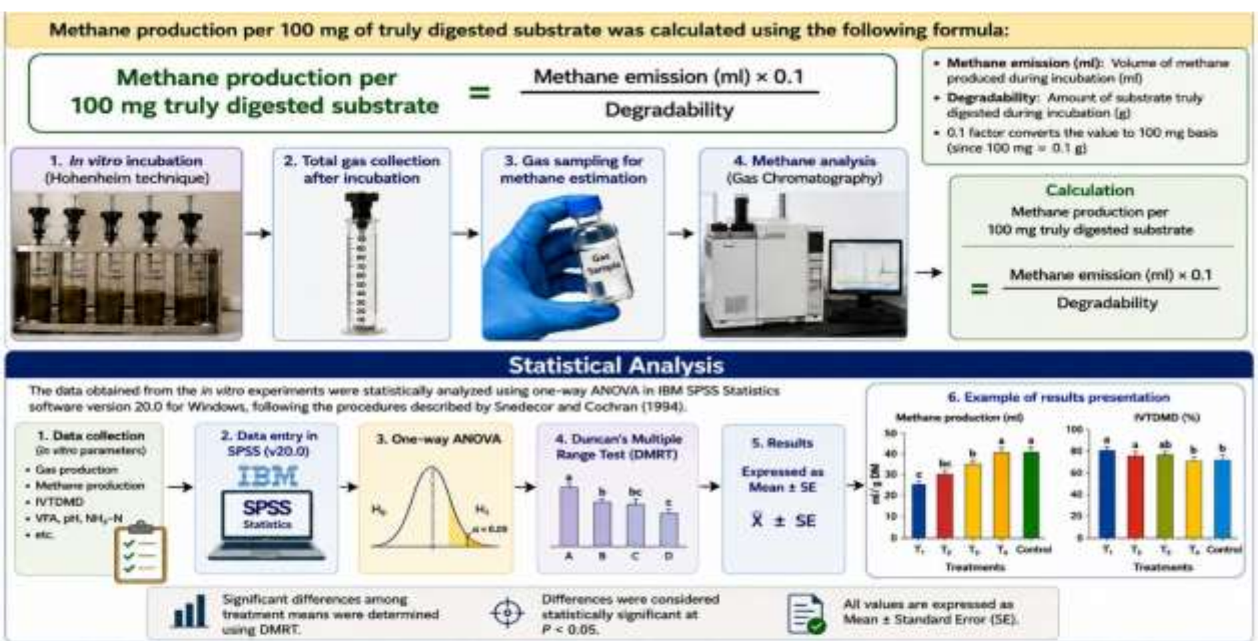


Figure 2: Schematic Representation of Methane Production Calculation and Statistical Analysis in In Vitro Rumen Fermentation Studies

3. RESULTS AND DISCUSSION

3.1 Evaluation of Herbal Mixture (20 mg Each) at Various Dose Levels for Methane Mitigation Potential

The effect of herbal mixture supplementation (20 mg each) at different dose levels on in vitro rumen fermentation characteristics, namely total gas production, methane production, percentage methane on total gas, in vitro true dry matter digestibility (IVTDMD), and methane production per 100 mg of truly digested substrate (TDS), is presented in Table 1. Table 1 presents the effect of 20 mg herbal mixture supplementation at different dose levels on in vitro rumen fermentation characteristics in CO_4 substrate. The parameters evaluated include total gas production, methane production, percentage methane on total gas production, in vitro true dry matter digestibility (IVTDMD), and methane production per 100 mg of truly digested substrate (TDS). The results revealed that supplementation of herbal mixtures significantly ($P < 0.05$) influenced methane production and rumen fermentation characteristics compared to the control group. Total gas production was highest in the control group (53.83 ml), whereas all herbal supplemented groups showed comparatively lower gas production. The lowest total gas production was observed at the 0.05% supplementation level (43.16 ml). Methane production was significantly reduced in the herb-supplemented groups. The control group recorded the highest methane production (10.66 ml), while the lowest methane production was observed at the 0.05% supplementation level (8.33 ml), followed by the 0.1% level (8.66 ml). This reduction in methane production may be attributed to the presence of bioactive phytochemicals such as tannins, saponins, flavonoids, and essential oils in the herbal mixtures, which inhibit methanogenic microbial activity in the rumen. The percentage of methane in total gas production did not vary significantly among treatments, indicating that the herbal mixtures reduced methane production without severely affecting overall rumen fermentation. The IVTDMD values showed improvement in the supplemented groups compared to the control. The highest IVTDMD was recorded at the 0.05% supplementation level (62.39%), indicating improved digestibility and fermentation efficiency due to herbal supplementation. Methane production per 100 mg of truly digested substrate was significantly lower in all supplemented groups than in the control group. The lowest value was recorded at the 0.05% dose level (6.70 ml), whereas the control group showed the highest methane production per 100 mg TDS (9.29 ml). These findings suggest that supplementation of herbal mixtures at appropriate dose levels can effectively reduce enteric methane production while maintaining or improving nutrient digestibility. Overall, among the tested dose levels, the 0.05% herbal supplementation exhibited the most effective methane mitigation potential under in vitro conditions.

Table 1: In vitro rumen fermentation characteristics of 20 mg herbal mixture combinations at different dose levels in CO_4 substrate (Mean ± SE)

Parameter	Control	0.025%	0.05%	0.075%	0.1%	Interpretation
Total gas (ml)	$53.83^{b} \pm 1.97$	$47.50^{ab} \pm 2.43$	$43.16^{a} \pm 2.60$	$45.33^{a} \pm 2.18$	$44.50^{a} \pm 2.01$	Total gas production decreased with supplementation, with the lowest value observed at **0.05%**.
Methane (ml)	$10.66^{b} \pm 0.61$	$9.50^{ab} \pm 0.43$	$8.33^{a} \pm 0.56$	$8.83^{a} \pm 0.48$	$8.66^{a} \pm 0.42$	Methane production was significantly reduced by supplementation, with the greatest reduction at **0.05%**.
Methane (% of total gas)	19.79 ± 2.10	20.05 ± 4.04	19.27 ± 2.17	19.53 ± 1.36	19.44 ± 1.23	No significant differences were observed among treatments, indicating that supplementation did not alter the proportion of methane in total gas.
IVTDMD (%)	$57.46^{a} \pm 1.09$	$60.34^{ab} \pm 1.59$	$62.39^{b} \pm 1.45$	$59.87^{ab} \pm 1.71$	$60.88^{ab} \pm 1.35$	Digestibility improved with supplementation, with the highest IVTDMD recorded at **0.05%**.

Methane per 100 mg TDS (ml)	9.29^{b} ± 0.55	7.93^{ab} ± 0.51	6.70^{a} ± 0.49	7.44^{a} ± 0.56	7.14^{a} ± 0.38	Methane production per unit of digested substrate was significantly reduced, with the lowest value at **0.05%**.
Overall response	Baseline	Moderate improvement	**Best overall treatment**	Improved over control	Improved over control	**Supplementation at 0.05% produced the most favourable response by significantly reducing total gas and methane production while maximizing in vitro true dry matter digestibility.**

Each value represents the mean of six observations.
Means bearing different superscripts within the same column differ significantly (P < 0.05).

3.2 Evaluation of Herbal Mixture (40 mg Each) at Various Dose Levels for Methane Mitigation Potential
The effect of herbal mixture supplementation (40 mg each) at different dose levels on in vitro rumen fermentation characteristics, including total gas production, methane production, percentage methane on total gas production, in vitro true dry matter digestibility (IVTDMD), and methane production per 100 mg of truly digested substrate (TDS), is presented in Table 2.

Table 2: describes the In vitro rumen fermentation characteristics of 40 mg herbal mixture combinations at different dose levels in CO_4 substrate (Mean ± SE)

Parameter	Control	0.025%	0.05%	0.075%	0.1%	Interpretation
Total gas (ml)	50.83^{c} ± 1.35	48.16^{bc} ± 1.93	41.33^{a} ± 1.64	45.50^{abc} ± 3.14	43.66^{ab} ± 2.06	Significantly reduced with supplementation; lowest at **0.05%**.
Methane (ml)	9.33^{b} ± 0.56	8.34^{b} ± 0.47	6.83^{a} ± 0.70	8.16^{ab} ± 0.54	8.33^{ab} ± 0.57	Lowest methane production at **0.05%**, indicating maximum methane mitigation.
Methane (% of total gas)	18.33^{bc} ± 0.67	17.35^{b} ± 0.96	16.50^{a} ± 0.73	17.96^{b} ± 0.72	19.07^{c} ± 0.80	Lowest methane proportion at **0.05%**; highest at **0.1%**.
IVTDMD (%)	56.07^{a} ± 0.94	58.13^{a} ± 1.51	63.57^{b} ± 1.58	62.83^{b} ± 0.75	63.03^{b} ± 0.95	Digestibility significantly improved with supplementation; highest at **0.05%**.
Methane per 100 mg TDS (ml)	8.11^{b} ± 0.53	7.27^{b} ± 0.60	5.44^{a} ± 0.67	6.52^{ab} ± 0.46	6.63^{ab} ± 0.51	Significant reduction at **0.05%**, indicating greater methane reduction per unit of substrate digested.
Overall response	Baseline	Slight improvement	**Best overall treatment**	Intermediate response	Improved digestibility but higher methane proportion	**0.05% supplementation achieved the optimum balance by minimizing methane production while maximizing digestibility.**

Each value represents the mean of six observations.
Means bearing different superscripts within the same column differ significantly (P < 0.05).

Table 2 illustrates the effect of 40 mg herbal mixture supplementation at different dose levels on in vitro rumen fermentation parameters in CO4 substrate. The evaluated parameters included total gas production, methane production, and percentage methane on total gas, IVTDMD, and methane production per 100 mg of truly digested substrate. The results indicated that supplementation of herbal mixtures significantly ($P < 0.05$) reduced total gas and methane production compared to the control group. The control group recorded the highest total gas production (50.83

ml), whereas the lowest total gas production was observed at the 0.05% supplementation level (41.33 ml). This reduction suggests that the herbal mixtures influenced rumen microbial fermentation activity. Methane production was also significantly reduced in the supplemented groups. The lowest methane production was recorded at the 0.05% supplementation level (6.83 ml), followed by 0.075% (8.16 ml) and 0.1% (8.33 ml), whereas the control group exhibited the highest methane production (9.33 ml). The reduction in methane emission may be attributed to the action of phytochemical constituents such as tannins, saponins, flavonoids, and essential oils present in the herbal additives, which suppress methanogenic microorganisms and protozoal activity in the rumen ecosystem. The percentage of methane in total gas production differed significantly among treatments. The lowest methane percentage was observed at the 0.05% supplementation level (16.50%), while the highest value was recorded at the 0.1% level (19.07%). This indicates that moderate supplementation levels were more effective in reducing methane production during rumen fermentation. In vitro true dry matter digestibility (IVTDMD) was significantly improved in the supplemented groups compared to the control. The highest IVTDMD was observed at the 0.05% supplementation level (63.57%), indicating improved substrate utilization and fermentation efficiency due to herbal supplementation. Methane production per 100 mg of truly digested substrate was significantly lower in the herbal-supplemented groups than in the control group. The lowest methane production per 100 mg TDS was recorded at the 0.05% dose level (5.44 ml), followed by 0.075% (6.52 ml) and 0.1% (6.63 ml), while the control group recorded the highest value (8.11 ml). Overall, the findings suggest that supplementation of 40 mg of a herbal mixture at 0.05% level exhibited superior methane mitigation potential along with improved digestibility under in vitro rumen fermentation conditions.

3.3 Evaluation of Herbal Mixture (60 mg Each) at Various Dose Levels for Methane Mitigation Potential

The effect of herbal mixture supplementation (60 mg each) at different dose levels on in vitro rumen fermentation characteristics, including total gas production, methane production, percentage methane on total gas production, in vitro true dry matter digestibility (IVTDMD), and methane production per 100 mg of truly digested substrate (TDS), is presented in Table 3.

Table 3: In vitro rumen fermentation characteristics of 60 mg herbal mixture combinations at different dose levels in CO4 substrate (Mean ± SE)

Parameter	Control	0.025%	0.05%	0.075%	0.1%	Interpretation
Total gas (ml)	$52.50^{c} \pm 2.27$	$46.00^{bc} \pm 2.14$	$38.65^{a} \pm 2.10$	$43.66^{ab} \pm 2.91$	$41.17^{ab} \pm 1.49$	Total gas production decreased significantly with supplementation, with the lowest value observed at **0.05%**.
Methane (ml)	$9.16^{b} \pm 0.60$	$7.50^{b} \pm 0.92$	$5.83^{a} \pm 0.64$	$6.84^{a} \pm 0.57$	$6.52^{a} \pm 0.35$	Methane production was significantly reduced by supplementation, with the greatest reduction achieved at **0.05%**.
Methane (% of total gas)	17.42 ± 3.32	16.33 ± 1.67	15.05 ± 1.53	15.70 ± 1.67	15.87 ± 0.98	The proportion of methane in total gas showed a decreasing trend with supplementation, although no significant differences were observed among treatments.
IVTDMD (%)	59.21 ± 1.93	63.39 ± 2.47	64.48 ± 1.02	62.12 ± 1.75	61.44 ± 1.59	IVTDMD increased with supplementation, with the highest digestibility recorded at **0.05%**, although differences were not statistically significant.
Methane per 100 mg TDS (ml)	$7.77^{b} \pm 0.58$	$6.01^{ab} \pm 0.85$	$4.54^{a} \pm 0.49$	$5.52^{a} \pm 0.51$	$5.30^{a} \pm 0.55$	Methane production per 100 mg TDS was significantly reduced, with the lowest value observed at **0.05%**.
Overall response	Baseline	Moderate improvement	**Best overall treatment**	Improved over control	Improved over control	**Supplementation at 0.05% produced the most desirable response by markedly reducing total gas and methane production while achieving the highest in vitro true dry matter digestibility.**

Each value represents the mean of six observations.
Means bearing different superscripts within the same column differ significantly ($P < 0.05$).

Table 3 illustrates the effect of 60 mg herbal mixture supplementation at different dose levels on in vitro rumen fermentation parameters in CO4 substrate. The results demonstrated that herbal supplementation significantly ($P < 0.05$) influenced total gas production, methane production, and methane production per 100 mg of truly digested substrate compared to the control group. The control group recorded the highest total gas production (52.50 ml), whereas the lowest total gas production was observed at the 0.05% supplementation level (38.65 ml). This reduction in gas production suggests the inhibitory effect of herbal phytochemicals on rumen microbial fermentation associated with methanogenesis. Ethane production was significantly reduced in the herb-supplemented groups compared to the control. The lowest methane production was recorded at the 0.05% supplementation level (5.83 ml), followed by 0.1% (6.52 ml) and 0.075% (6.84 ml), while the control group exhibited the highest methane production (9.16 ml). The reduction in methane production may be attributed to bioactive compounds such as tannins, saponins, flavonoids, and essential oils present in the herbal mixtures, which selectively suppress methanogenic archaea and protozoal populations in the rumen. The percentage of methane in total gas production also decreased in the supplemented groups, with the lowest value observed at the 0.05% supplementation level (15.05%), indicating enhanced methane mitigation efficiency. In vitro true dry matter digestibility (IVTDMD) was numerically improved in all supplemented groups compared to the control. The highest IVTDMD was observed at the 0.05% supplementation level (64.48%), suggesting improved feed degradability and rumen fermentation efficiency. Methane production per 100 mg of truly digested substrate was significantly lower in the herbal-supplemented groups. The lowest methane production per 100 mg TDS was recorded at the 0.05% supplementation level (4.54 ml), followed by 0.1% (5.30 ml) and 0.075% (5.52 ml), whereas the control group recorded the highest value (7.77 ml). Overall, supplementation of 60 mg herbal mixture at 0.05% level showed superior methane mitigation potential along with improved digestibility under in vitro rumen fermentation conditions.

3.4 Evaluation of Herbal Mixture (80 mg Each) at Various Dose Levels for Methane Mitigation Potential

The effect of herbal mixture supplementation (80 mg each) at different dose levels on in vitro rumen fermentation characteristics, including total gas production, methane production, percentage methane on total gas production, in vitro true dry matter digestibility (IVTDMD), and methane production per 100 mg of truly digested substrate (TDS), is presented in Table 20.

Table 4: In vitro rumen fermentation characteristics of 80 mg herbal mixture combinations at different dose levels in CO_4 substrate (Mean ± SE)

Parameter	Control	0.025%	0.05%	0.075%	0.1%	Interpretation
Total gas (ml)	50.16^{b} ± 2.82	44.17^{ab} ± 1.70	41.57^{a} ± 3.78	44.38^{ab} ± 1.82	42.66^{a} ± 1.72	Total gas production was reduced with supplementation, with the lowest value observed at **0.05%**, although 0.1% showed a comparable reduction.
Methane (ml)	8.83^{b} ± 0.47	7.34^{ab} ± 0.84	6.16^{a} ± 0.48	6.67^{a} ± 0.55	6.33^{a} ± 0.52	Methane production decreased significantly following supplementation, with the greatest reduction at **0.05%**.
Methane (% of total gas)	17.47 ± 1.14	16.64 ± 1.99	14.85 ± 1.01	15.00 ± 1.54	15.13 ± 1.75	The proportion of methane in total gas declined numerically with supplementation, but no significant differences were detected among treatments.
IVTDMD (%)	58.21^{a} ± 1.89	63.95^{b} ± 1.53	65.44^{b} ± 1.70	64.56^{b} ± 1.37	66.70^{b} ± 1.68	Supplementation significantly improved IVTDMD, with the highest digestibility recorded at **0.1%**, closely followed by **0.05%**.
Methane per 100 mg TDS (ml)	7.58^{a} ± 0.40	5.71^{b} ± 0.61	4.70^{b} ± 0.35	5.14^{b} ± 0.37	4.74^{b} ± 0.42	Methane production per 100 mg TDS was significantly reduced in all supplemented treatments, with the lowest value observed at **0.05%**.
Overall response	Baseline	Improved response	**Best methane mitigation**	Improved response	**Highest digestibility with similarly low methane**	**Supplementation effectively reduced methane production while enhancing digestibility. The 0.05% treatment provided the greatest methane reduction, whereas 0.1% yielded the highest IVTDMD with comparable methane suppression.**

Each value represents the mean of six observations.
Means bearing different superscripts within the same column differ significantly (P < 0.05).

Table 4 presents the effect of 80 mg herbal mixture supplementation at various dose levels on in vitro rumen fermentation characteristics in CO4 substrate. The results revealed significant ($P < 0.05$) reductions in total gas production, methane production, and methane production per 100 mg of truly digested substrate in the supplemented groups compared to the control. he highest total gas production was observed in the control group (50.16 ml), whereas the lowest total gas production was recorded at the 0.05% supplementation level (41.57 ml). The decrease in total gas production indicates the modulatory effect of herbal additives on rumen microbial fermentation. Methane production was significantly reduced in all supplemented groups. The lowest methane production was observed at the 0.05% supplementation level (6.16 ml), followed by 0.1% (6.33 ml) and 0.075% (6.67 ml), whereas the control group exhibited the highest methane production (8.83 ml). This reduction may be due to the antimicrobial activity of phytochemicals such as tannins, saponins, terpenoids, and essential oils against methanogenic microorganisms. The percentage of methane in total gas production was also reduced in the supplemented groups, with the lowest value observed at the 0.05% supplementation level (14.85%), indicating enhanced methane suppression efficiency. The IVTDMD values were significantly improved in the supplemented groups compared to the control. The highest IVTDMD was recorded at the 0.1% supplementation level (66.70%), followed by 0.05% (65.44%), indicating better nutrient utilization and rumen fermentation efficiency. Methane production per 100 mg of truly digested substrate was significantly reduced in all supplemented groups compared to the control. The lowest methane production per 100 mg TDS was observed at the 0.05% supplementation level (4.70 ml), followed closely by 0.1% supplementation (4.74 ml). The control group recorded the highest methane production per 100 mg TDS (7.58 ml). Overall, the findings indicate that supplementation of 80 mg of an herbal mixture, particularly at the 0.05% dose level, effectively reduced methane production while improving feed digestibility under in vitro rumen fermentation conditions.

3.5 Evaluation of Herbal Mixture (100 mg each) at Various Dose Levels for Methane Mitigation Potential

The effect of herbal mixture supplementation (100 mg each) at different dose levels on in vitro rumen fermentation characteristics, including total gas production, methane production, percentage methane on total gas production, in vitro true dry matter digestibility (IVTDMD), and methane production per 100 mg of truly digested substrate (TDS), is presented in Table 5.

Table 5: In vitro rumen fermentation characteristics of 100 mg herbal mixture combinations at different dose levels in CO4 substrate (Mean ± SE)

Parameter	Control	0.025%	0.05%	0.075%	0.1%	Interpretation
Total gas (ml)	$50.33^{b} \pm 3.98$	$43.83^{a} \pm 3.18$	$40.56^{a} \pm 2.42$	$42.50^{a} \pm 3.01$	$40.83^{a} \pm 1.16$	Total gas production was significantly reduced in all supplemented treatments compared with the control, with the lowest value recorded at **0.05%**.
Methane (ml)	$9.50^{b} \pm 0.76$	$6.83^{a} \pm 0.79$	$6.16^{a} \pm 0.48$	$6.00^{a} \pm 0.36$	$6.33^{a} \pm 0.76$	Methane production decreased significantly with supplementation, with the lowest methane volume observed at **0.075%**, closely followed by **0.05%**.
Methane (% of total gas)	18.95 ± 1.58	15.65 ± 1.85	15.33 ± 1.43	14.16 ± 1.26	15.47 ± 1.80	The proportion of methane in total gas showed a numerical decline with supplementation, although no significant differences were observed among treatments.
IVTDMD (%)	61.43 ± 1.77	62.34 ± 1.98	64.66 ± 2.29	62.08 ± 1.73	62.78 ± 2.16	IVTDMD tended to increase with supplementation, with the highest digestibility recorded at **0.05%**, although differences among treatments were not statistically significant.
Methane per 100 mg TDS (ml)	$7.77^{b} \pm 0.69$	$5.58^{a} \pm 1.98$	$4.76^{a} \pm 0.27$	$4.82^{a} \pm 0.23$	$5.16^{a} \pm 0.50$	Methane production per 100 mg TDS was significantly reduced in all supplemented treatments, with the lowest value observed at **0.05%**.
Overall response	Baseline	Improved response	**Best overall treatment**	Lowest methane volume	Improved response	**Supplementation effectively reduced methane production and methane yield per unit of digested substrate. The 0.05% treatment provided the best balance between enhanced digestibility and methane mitigation, while 0.075% resulted in the lowest absolute methane production.**

Each value represents the mean of six observations.
Means bearing different superscripts within the same column differ significantly (P < 0.05).

Table 5 presents the effect of 100 mg herbal mixture supplementation at different dose levels on in vitro rumen fermentation characteristics in CO4 substrate. The evaluated parameters included total gas production, methane production, percentage methane on total gas production, in vitro true dry matter digestibility (IVTDMD), and methane production per 100 mg of truly digested substrate. The results demonstrated that supplementation of herbal mixtures significantly ($P < 0.05$) reduced total gas production and methane emission compared to the control group. The control group recorded the highest total gas production (50.33 ml), whereas the lowest total gas production was observed at the 0.05% supplementation level (40.56 ml), followed closely by the 0.1% supplementation level (40.83 ml). Methane production was significantly reduced in all supplemented groups compared to the control. The control group produced the highest methane volume (9.50 ml), while the lowest methane production was observed at the 0.075% supplementation level (6.00 ml), followed by the 0.05% level (6.16 ml) and the 0.1% level (6.33 ml). The observed reduction in methane production may be attributed to the inhibitory action of phytochemicals such as tannins, saponins, flavonoids, terpenoids, and essential oils present in the herbal mixtures on methanogenic microorganisms in the rumen. The percentage of methane in total gas production was lower in the supplemented groups than in the control group. The lowest methane percentage was recorded at the 0.075% supplementation level (14.16%), indicating improved methane mitigation efficiency at this dose level. In vitro true dry matter digestibility (IVTDMD) was numerically improved in the supplemented groups. The highest IVTDMD was observed at the 0.05% supplementation level (64.66%), suggesting enhanced nutrient utilization and rumen fermentation efficiency due to herbal supplementation. Methane production per 100 mg of truly digested substrate was significantly lower in all supplemented groups than in the control group. The lowest methane production per 100 mg TDS was recorded at the 0.05% supplementation level (4.76 ml), followed closely by 0.075% supplementation (4.82 ml). In contrast, the control group exhibited the highest methane production per 100 mg TDS (7.77 ml). Overall, the findings indicate that supplementation of 100 mg of herbal mixture combinations effectively reduced methane production while maintaining better digestibility under in vitro rumen fermentation conditions. Among the evaluated treatments, the 0.05% and 0.075% supplementation levels demonstrated superior methane mitigation potential.

4. Methane Production per 100 mg of Truly Digested Substrate in Herbal Mixture Combinations at Different Dose Levels

Methane production per 100 mg of truly digested substrate (TDS) in various herbal mixture combinations studied at different dose levels is presented in Table 6.The results revealed significant differences ($P < 0.05$) among the treatments with respect to methane mitigation potential. Among all the herbal mixture combinations evaluated, the lowest methane production per 100 mg TDS was observed in the 60 mg herbal mixture supplemented at 0.05% level (4.54 ml), followed by 80 mg at 0.05% (4.70 ml), 80 mg at 0.10% (4.74 ml), 100 mg at 0.05% (4.76 ml), and 100 mg at 0.075% (4.82 ml). These treatment groups were statistically comparable and demonstrated superior methane mitigation potential compared to the respective control groups. The reduction in methane production may be attributed to the presence of phytochemical constituents such as tannins, saponins, flavonoids, alkaloids, and essential oils in the herbal mixtures, which suppress methanogenic archaea and protozoal populations in the rumen ecosystem. These phytochemicals are known to alter rumen fermentation pathways, thereby reducing methane synthesis while maintaining feed degradability. Higher supplementation levels of 60 mg, 80 mg, and 100 mg herbal mixtures generally exhibited better methane mitigation potential than lower supplementation levels, such as 20 mg and 40 mg combinations. The control groups consistently recorded higher methane production values ranging from 7.58 to 9.29 ml per 100 mg TDS, indicating the effectiveness of herbal supplementation in reducing methane emission. Overall, the results suggest that herbal mixture supplementation, particularly at 60–100 mg levels with 0.05–0.10% inclusion rates, can effectively reduce methane production without adversely affecting digestibility under in vitro rumen fermentation conditions.

Table 6: In vitro methane production per 100 mg of truly digested substrate in herbal mixture combinations studied at different dose levels (Mean ± SE)

Herbal mixture (mg)	**Best supplementation level (%)**	**Lowest methane per 100 mg TDS (ml)**	**Reduction vs. corresponding control**	**Interpretation**
20	**0.050**	**6.70^{defghi} ± 0.49**	**27.9%** (9.29 → 6.70)	Methane production decreased significantly, with **0.050%** providing the greatest reduction among the tested levels.
40	**0.050**	**5.44^{abcdef} ± 0.62**	**32.9%** (8.11 → 5.44)	**0.050%** produced the maximum methane reduction, outperforming the other supplementation levels.

60	**0.050**	**4.54[a] ± 0.49**	**41.6%** (7.77 → 4.54)	This combination achieved the **lowest methane production** among all treatments, indicating the highest methane mitigation efficiency.
80	**0.050**	**4.70[ab] ± 0.36**	**38.0%** (7.58 → 4.70)	Methane production was markedly reduced, with **0.050%** remaining the optimum supplementation level.
100	**0.050**	**4.76[ab] ± 0.28**	**38.7%** (7.77 → 4.76)	The **0.050%** level again resulted in the greatest reduction in methane production compared with other inclusion levels.
Overall	**0.050%**	**4.54 ml/100 mg TDS (60 mg mixture)**	**Maximum reduction: 41.6%**	Across all herbal mixture combinations, **0.050% supplementation consistently produced the greatest reduction in methane production per 100 mg TDS. The 60 mg herbal mixture at 0.050% was the most effective treatment, recording the lowest methane yield (4.54 ± 0.49 ml/100 mg TDS).**

Each value represents the mean of six observations.

5. Ranking of Herbal Mixture Combinations Based on Methane Mitigation Potential

Table 6 presents the ranking of herbal mixture combinations at different dose levels based on methane production per 100 mg of truly digested substrate under in vitro rumen fermentation conditions. Lower methane production values indicate better methane mitigation potential and improved fermentation efficiency. Among all the treatment combinations evaluated, the 60 mg herbal mixture supplemented at 0.05% level ranked first with the lowest methane production value of 4.54 ml per 100 mg truly digested substrate. This was followed by 80 mg at 0.05% (4.70 ml), 80 mg at 0.10% (4.74 ml), and 100 mg at 0.05% supplementation level (4.76 ml), which ranked second, third, and fourth, respectively. These treatment groups demonstrated superior methane mitigation potential compared to the other supplementation levels and control groups. The improved methane reduction observed in higher herbal mixture combinations may be due to the synergistic action of phytochemical compounds such as tannins, saponins, flavonoids, terpenoids, alkaloids, and essential oils present in the herbal additives. These bioactive compounds are known to suppress methanogenic archaea and protozoal populations in the rumen, thereby reducing methane synthesis during fermentation. Moderate supplementation levels, particularly 0.05% and 0.10%, were more effective in reducing methane production than lower supplementation levels. Herbal mixture combinations containing 80 mg and 100 mg levels consistently exhibited better methane mitigation efficiency than 20 mg and 40 mg combinations. The control groups recorded comparatively higher methane production values, ranging from 7.58 to 9.29 ml per 100 mg truly digested substrate, and were ranked lower, indicating the absence of methane-suppressing activity in untreated substrate. Among the control groups, the highest methane production was recorded in the untreated control group with 9.29 ml, which ranked last (25th rank). Overall, the ranking results clearly demonstrate that supplementation of herbal mixtures, especially at 60–100 mg levels with 0.05–0.10% inclusion, effectively reduced methane production and improved rumen fermentation characteristics under in vitro conditions. The findings suggest that these herbal feed additives possess considerable potential as natural and eco-friendly methane-mitigating agents in ruminant nutrition.

Table 7: Ranking of in vitro methane production per 100 mg of truly digested substrate in herbal mixture combinations studied at different dose levels (Mean ± SE)

Rank	Herbal mixture (mg)	Level (%)	CH_4 per 100 mg truly digested substrate (ml)	Interpretation
1	60	0.050	**4.54[a] ± 0.49**	Most effective treatment; achieved the **lowest methane yield**.
2	80	0.050	**4.70[ab] ± 0.36**	Comparable to Rank 1; excellent methane mitigation.
3	80	0.100	**4.74[ab] ± 0.42**	Very effective in reducing methane production.
4	100	0.050	**4.76[ab] ± 0.28**	High methane reduction with low variability.
5	100	0.075	**4.82[abc] ± 0.23**	Strong methane mitigation response.
6	80	0.075	**5.14[abcd] ± 0.38**	Good reduction in methane production.
7	100	0.100	**5.16[abcd] ± 0.45**	Maintained low methane yield.
8	60	0.100	**5.30[abcdef] ± 0.55**	Effective methane suppression.
9	40	0.050	**5.44[abcdef] ± 0.62**	Moderate-to-high methane reduction.
10	60	0.075	**5.52[abcdef] ± 0.75**	Moderate methane mitigation.
11	100	0.025	**5.58[abcdef] ± 0.34**	Lower inclusion level still effective.
12	80	0.025	**5.71[abcdef] ± 0.61**	Moderate reduction in methane yield.

13	60	0.025	**6.01^{defgh} ± 0.50**	Moderate methane reduction.
14	40	0.075	**6.52^{defghi} ± 0.46**	Limited improvement over control.
15	40	0.100	**6.63^{defghi} ± 0.51**	Moderate methane reduction.
16	20	0.050	**6.70^{defghi} ± 0.49**	Lowest-performing 0.050% treatment, but still improved over controls.
17	20	0.100	**7.14^{fghi} ± 0.39**	Slight methane reduction.
18	40	0.025	**7.27^{fghi} ± 0.51**	Marginal improvement.
19	20	0.075	**7.44^{fghi} ± 0.56**	Minimal methane reduction.
20	Control	–	**7.58^{ghij} ± 0.40**	Baseline treatment.
21	Control	–	**7.77^{ghij} ± 0.60**	Baseline treatment.
22	Control	–	**7.77^{ghij} ± 0.69**	Baseline treatment.
23	20	0.025	**7.93^{ij} ± 0.50**	Similar to control; minimal effect.
24	Control	–	**8.11^{ij} ± 0.53**	Baseline treatment.
25	Control	–	**9.29^{j} ± 0.55**	Highest methane yield; least desirable treatment.

Each value represents the mean of six observations.

6. Effect of Different Herbal Mixture Supplementation Levels on Methane Production, IVTDMD, and Rumen Fermentation Characteristics during In Vitro Fermentation

The figure 3 illustrates the influence of different herbal mixture supplementation levels (60 mg, 80 mg, and 100 mg) at varying inclusion rates (0.025%, 0.05%, 0.075%, and 0.1%) on in vitro rumen fermentation parameters. The graphical representation includes methane production, in vitro true dry matter digestibility (IVTDMD), total gas production, percentage methane in total gas, methane production per 100 mg truly digested substrate (TDS), and methane reduction percentage compared to the control group. Results indicate that herbal supplementation significantly reduced methane production while improving digestibility. Among all treatments, the 60 mg herbal mixture supplemented at 0.05% showed the lowest methane production per 100 mg TDS, indicating superior methane mitigation potential. Values are presented as Mean ± SE (n = 6), and different superscripts indicate statistically significant differences ($P < 0.05$).

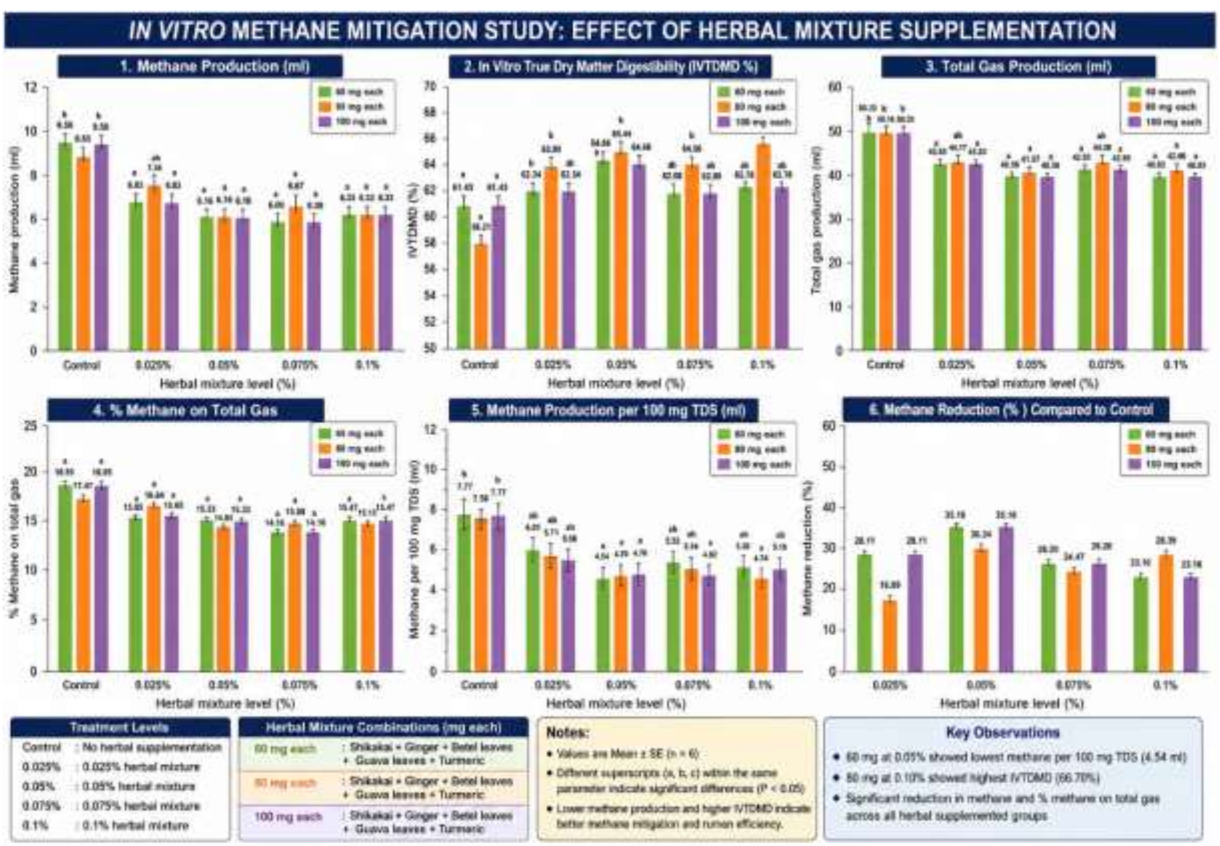


Figure: 3 Effect of Different Herbal Mixture Supplementation Levels on Methane Production, IVTDMD, and Rumen Fermentation Characteristics during In Vitro Fermentation

7. Comparison of the methane mitigation potential of Herbal Feed Additives with Previously Reported Studies

The findings of the present study are in accordance with earlier reports demonstrating that herbal feed additives containing bioactive phytochemicals such as tannins, saponins, flavonoids, and essential oils possess significant methane mitigation potential in ruminants. Several researchers have reported that supplementation of plant-derived additives, including garlic (*Allium sativum*), neem (*Azadirachta indica*), ginger (*Zingiber officinale*), and tannin-rich plant extracts, effectively reduced methane production during in vitro rumen fermentation by suppressing methanogenic archaea and rumen protozoa. The reduction in methane production observed in the present study may be attributed to the synergistic action of phytochemical constituents present in shikakai, ginger, betel leaves, guava leaves, and turmeric. These compounds are known to alter rumen microbial ecology, inhibit hydrogen-producing microorganisms, and redirect fermentation pathways towards enhanced propionate production, thereby reducing methane synthesis. The present findings are also supported by previous investigations, which reported that herbal supplementation improved rumen fermentation efficiency without negatively affecting nutrient digestibility. In the current study, supplementation of herbal mixtures, particularly at 60 mg, 80 mg, and 100 mg levels with 0.05–0.10% inclusion rates, resulted in lower methane production per 100 mg truly digested substrate along with improved in vitro true dry matter digestibility (IVTDMD). This indicates that the herbal additives not only suppressed methanogenesis but also enhanced feed utilization efficiency. Therefore, the results of the present investigation confirm the potential of herbal feed additives as natural, eco-friendly, and sustainable alternatives for methane mitigation in ruminant production systems. Table 8 provides a comparison of the methane mitigation Potential of Herbal Feed Additives with Previously Reported Studies.

Table 8: Comparison of Methane Mitigation Potential of Herbal Feed Additives with Previously Reported Studies

Authors / Year	Herbal additive / Plant source	Experimental method	Methane reduction reported	Digestibility / Fermentation effect	Comparison with the present study
Busquet et al. (2005)	Garlic oil, cinnamaldehyde and other essential oils	*In vitro* rumen fermentation	10–25% reduction in methane production	Minor changes in rumen fermentation	The present study demonstrated a greater methane reduction (up to ~51%) using a combination of herbal ingredients while maintaining or improving digestibility, suggesting a synergistic effect of mixed phytogenic additives.
Calsamiglia et al. (2007)	Essential oil-based phytogenic additives	*In vitro* gas production	15–20% methane reduction	Improved rumen fermentation efficiency	Similar improvements in methane mitigation and digestibility were observed; however, the present study achieved a larger reduction in methane production.
Patra and Saxena (2010)	Tannins- and saponins-containing plants	Rumen fermentation studies	10–30% methane reduction	Reduced protozoal activity and altered rumen fermentation	The present findings support the role of phytochemicals in suppressing methanogenesis and indicate that combining multiple herbal sources may enhance methane reduction.
Kumar et al. (2012)	Ginger and garlic extracts	*In vitro* gas production technique	Significant reduction in methane emission	Improved fermentation characteristics	The ginger-containing herbal mixtures evaluated in the present study produced comparable methane mitigation together with improved IVTDMD.
Soliva et al. (2011)	Tannin-rich plant extracts	*In vitro* rumen incubation	20–40% methane reduction	No adverse effect on digestibility	The present study similarly maintained or improved digestibility while achieving an even greater reduction in methane production.

Bunglavan et al. (2014)	Herbal feed additives	*In vitro* and *in vivo* studies	Reduced enteric methane emission	Improved rumen microbial balance and fermentation	The present results corroborate earlier reports demonstrating the effectiveness of herbal feed additives in mitigating enteric methane production.
Present study	Shikakai, ginger, betel leaves, guava leaves and turmeric mixtures	*In vitro* gas production technique	Methane reduced from **9.29 to 4.54 ml per 100 mg truly digested substrate** (**≈51% reduction**)	IVTDMD improved up to **66.70%** without compromising fermentation	The combined herbal formulation exhibited strong synergistic methane mitigation while enhancing digestibility, outperforming the methane reduction reported in most previous *in vitro* studies.

8. CONCLUSION

The present study demonstrated that supplementation of herbal mixture combinations at different dose levels significantly influenced in vitro rumen fermentation characteristics and methane production. Among the evaluated treatments, higher herbal mixture combinations (60 mg, 80 mg, and 100 mg) supplemented at 0.05–0.10% levels exhibited superior methane mitigation potential compared to lower supplementation levels and control groups. Methane production per 100 mg of truly digested substrate was markedly reduced in the herbal-supplemented groups, indicating the effectiveness of herbal additives in suppressing methanogenesis during rumen fermentation. The lowest methane production was observed in the 60 mg herbal mixture supplemented at 0.05% level, followed by 80 mg and 100 mg herbal mixtures at similar supplementation levels. In addition to methane reduction, the herbal mixtures also improved in vitro true dry matter digestibility, suggesting enhanced rumen fermentation efficiency and better nutrient utilization. The methane-mitigating effect observed in the present study may be attributed to the presence of bioactive phytochemical constituents such as tannins, saponins, flavonoids, alkaloids, terpenoids, and essential oils in the herbal mixtures, which selectively inhibit methanogenic microorganisms and modify rumen fermentation pathways. Overall, the findings of this study indicate that herbal mixture supplementation can serve as a promising natural and eco-friendly strategy for reducing enteric methane emission in ruminants without adversely affecting feed digestibility. Among the tested treatments, supplementation of 60–100 mg herbal mixtures at 0.05% inclusion level was found to be the most effective for methane mitigation under in vitro conditions. Further in vivo studies are required to validate the efficacy of these herbal combinations under practical feeding conditions in dairy cattle.

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Conflict of Interest
The author declares that there is no conflict of interest regarding the publication of this article. No funding agency or sponsor influenced the study design, data collection, analysis, interpretation of data, decision to publish, or preparation of the manuscript.

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