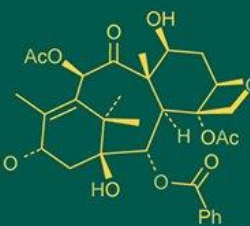
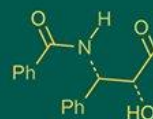
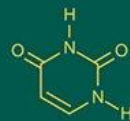


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
IJABR 2026; 10(6): 819-829
www.biochemjournal.com
Received: 04-04-2026
Accepted: 08-05-2026

R Balamurugan
Associate Professor,
Department of Animal
Nutrition, Veterinary College
and Research Institute
Veerapandi, Theni District,
Tamil Nadu, India

Methane mitigation potential of herbal mixture supplementation in total mixed rations: Effects on *in vitro* fermentation characteristics, rumen protozoa, and methanogenic archaea

R Balamurugan

DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i6j.8763>

Abstract

The present study was conducted to evaluate the methane mitigation potential of herbal mixture combinations incorporated into Total Mixed Rations (TMR) using *in vitro* gas production and quantitative PCR (qPCR) techniques. Initially, nine promising herbal mixture combinations identified from previous screening studies were evaluated at different dose levels for their effects on methane production, fermentation characteristics, microbial biomass, and rumen protozoal population. Among the tested combinations, supplementation of the herbal mixture at 60 mg (0.05%) significantly reduced methane production (5.16 ml), methane percentage in total gas (19.00%), and methane production per 100 mg truly digested substrate (3.23 ml) compared with the control (9.16 ml, 22.34%, and 5.52 ml, respectively; $p < 0.05$), without adversely affecting *in vitro* true dry matter digestibility (IVTDMD). This treatment also decreased ammonia nitrogen concentration and total protozoal count while enhancing microbial biomass production. Based on these findings, the 60 mg herbal mixture at 0.05% inclusion level was selected for further validation and compared with 0.04% and 0.06% inclusion levels. Both 0.05% and 0.06% supplementation significantly reduced methane production and methane per unit of truly digested substrate compared with the control ($p < 0.05$), while maintaining comparable IVTDMD, pH, total volatile fatty acids, and microbial biomass. Furthermore, qPCR analysis revealed a significant reduction in the abundance of whole methanogens, Methanobacteriales, Methanosarcinales, and Methanomicrobiales in herbal mixture-supplemented groups, particularly at 0.05% and 0.06% inclusion levels. The reduction in methanogenic archaea was accompanied by a decrease in rumen protozoal population. The selected herbal mixture supplemented at 60 mg (0.05%) reduced methane production by 43.67%, methane per 100 mg truly digested substrate by 41.49%, and protozoal population by 40.92% compared with the control. "The study concludes that supplementation of the herbal mixture at 60 mg (0.05%) in TMR effectively mitigates methane production by modulating rumen methanogens and protozoa without compromising feed digestibility and fermentation characteristics. Therefore, the herbal mixture may serve as a promising natural feed additive for reducing enteric methane emissions from ruminants.

Keywords: Herbal mixture, methane mitigation, total mixed ration, *in vitro* gas production, rumen methanogens, QPCR, methanogenic archaea, protozoa, microbial biomass, enteric methane emission, rumen fermentation, feed additive

1. Introduction

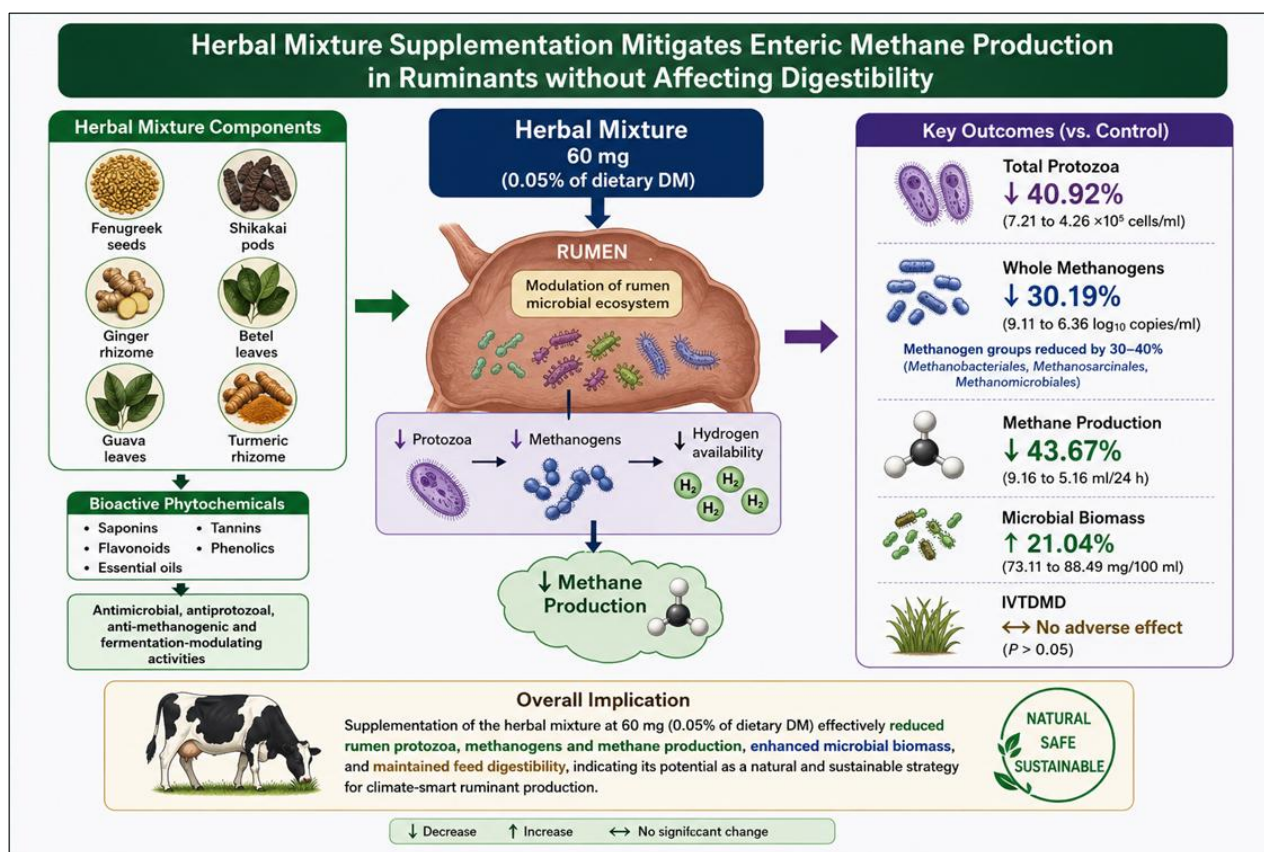
Livestock production plays a vital role in global food security by providing milk, meat, fiber, and livelihood opportunities to millions of people worldwide. Among livestock species, ruminants contribute substantially to the agricultural economy through their ability to convert fibrous feed resources into valuable animal products. However, enteric methane production during ruminal fermentation has emerged as a major environmental concern due to its contribution to greenhouse gas emissions and climate change. Methane possesses a global warming potential approximately 28 times greater than carbon dioxide over a 100-year period and accounts for a significant proportion of greenhouse gases emitted from the livestock sector. Methane production in the rumen is a natural consequence of anaerobic fermentation, where methanogenic archaea utilize hydrogen and carbon dioxide generated during microbial degradation of feed. Although methanogenesis serves as a hydrogen sink that maintains rumen fermentation, it represents an energy loss of approximately 2-12% of

Corresponding Author:
R Balamurugan
Associate Professor,
Department of Animal
Nutrition, Veterinary College
and Research Institute
Veerapandi, Theni District,
Tamil Nadu, India

gross dietary energy consumed by the animal. Therefore, reducing enteric methane emissions not only contributes to environmental sustainability but also improves feed energy utilization and animal productivity. Several methane mitigation strategies have been investigated, including dietary manipulation, chemical inhibitors, ionophores, probiotics, vaccines, and plant-derived bioactive compounds. Among these approaches, phytogetic feed additives have gained considerable attention because of their natural origin, consumer acceptability, and minimal risk of chemical residues in animal products.

Herbs and their bioactive constituents such as tannins, saponins, flavonoids, essential oils, and phenolic compounds have been reported to suppress methanogenic archaea, reduce ruminal protozoal populations, and alter fermentation pathways, thereby decreasing methane production. The use of herbal mixtures rather than individual herbs may provide synergistic effects due to the complementary actions of diverse phytochemicals. Previous studies have demonstrated that certain herbal combinations can effectively reduce methane production while maintaining normal rumen fermentation and nutrient digestibility. However, the efficacy of herbal mixtures depends on their composition, inclusion level, and interaction with rumen microbial populations. Therefore,

identifying optimum combinations and dose levels is essential before recommending their practical application in ruminant feeding systems. *In vitro* gas production techniques provide a rapid, economical, and reliable method for screening feed additives for their methane mitigation potential. Furthermore, advances in molecular biology, particularly quantitative polymerase chain reaction (qPCR), have enabled precise quantification of specific rumen microbial populations involved in methanogenesis and fiber degradation, thereby improving the understanding of mechanisms underlying methane reduction. The present study was undertaken to evaluate the methane mitigation potential of selected herbal mixture combinations incorporated into total mixed rations using *in vitro* gas production techniques. The study further aimed to identify the optimum inclusion level of the most promising herbal mixture and to investigate its effects on rumen fermentation characteristics, protozoal population, microbial biomass production, and the abundance of methanogenic archaea and fibrolytic bacteria using qPCR analysis. The findings are expected to contribute toward the development of sustainable and environmentally friendly feeding strategies for reducing enteric methane emissions from ruminants without compromising rumen function and feed utilization.



2. Materials and Methods

2.1 Experimental design

The present study was conducted to evaluate the methane mitigation potential of selected herbal mixture combinations incorporated into total mixed rations (TMR) using the *in vitro* gas production technique. Herbal mixture combinations that exhibited superior methane reduction potential in previous *in vitro* screening studies were selected for further evaluation in TMR. The investigation was carried out in three successive experiments.

Experiment 1: Evaluation of potential herbal mixture combinations in total mixed rations

Nine promising herbal mixture combinations identified from previous experiments were incorporated into total mixed rations and evaluated for their methane mitigation potential using the *in vitro* gas production technique. The treatments consisted of:

- Control (without herbal supplementation)
- Herbal mixture 60 mg (0.05%)
- Herbal mixture 80 mg (0.05%)

- Herbal mixture 100 mg (0.05%)
- Herbal mixture 100 mg (0.075%)
- Herbal mixture 80 mg (0.075%)
- Herbal mixture 40 mg (0.05%)
- Herbal mixture 60 mg (0.075%)
- Herbal mixture 100 mg (0.025%)
- Herbal mixture 80 mg (0.025%)

The effects of these treatments on total gas production, methane production, percentage methane in total gas, *in vitro* true dry matter digestibility (IVTDMD), methane production per 100 mg truly digested substrate (TDS), pH, ammonia nitrogen, total volatile fatty acids (TVFA), microbial biomass production, and total protozoal count were evaluated.

Experiment 2: Validation of the most effective herbal mixture combination

Based on the results of Experiment IV, the herbal mixture combination supplemented at 60 mg (0.05%) level, which exhibited the lowest methane production per 100 mg truly digested substrate without adversely affecting digestibility, was selected for further validation.

The selected herbal mixture was evaluated at three inclusion levels:

- 60 mg (0.04%)
- 60 mg (0.05%)
- 60 mg (0.06%) along with an unsupplemented control.

The effects on total gas production, methane production, percentage methane in total gas, IVTDMD, methane production per 100 mg TDS, pH, ammonia nitrogen, TVFA concentration, microbial biomass production, and total protozoal count were studied.

Experiment 3: Quantification of rumen methanogens and fibrolytic bacteria by qPCR

Based on the findings of Experiment V, rumen microbial populations were quantified in the following treatments:

- Control
- Herbal mixture 60 mg (0.04%)
- Herbal mixture 60 mg (0.05%)
- Herbal mixture 60 mg (0.06%)

Quantitative polymerase chain reaction (qPCR) was employed to determine the abundance of selected methanogenic archaea and fibrolytic bacteria.

• Preparation of total mixed ration and herbal mixture supplementation

The basal substrate consisted of a total mixed ration formulated according to the nutrient requirements of ruminants. Feed ingredients were dried, ground through a 1-mm sieve, and thoroughly mixed to obtain a homogeneous substrate. The selected herbal mixture combinations were incorporated into the total mixed ration at the respective dose levels prior to incubation. Herbal supplementation levels were expressed as percentages of substrate dry matter.

• Collection of rumen inoculum

Rumen liquor was collected before the morning feeding from healthy adult donor ruminants maintained on a standard feeding regimen. The rumen contents were strained through four layers of muslin cloth under continuous flushing with carbon dioxide to maintain anaerobic conditions. The filtered rumen liquor was transferred immediately to pre-warmed insulated flasks and transported to the laboratory for use as inoculum.

In vitro gas production technique

The *in vitro* gas production study was conducted following the procedure of Menke and Steingass (1988) [17]. Approximately 500 mg of substrate was weighed into calibrated fermentation syringes.

The incubation medium consisted of buffered mineral solution and rumen liquor mixed under continuous carbon dioxide flushing to maintain anaerobic conditions. The substrate and corresponding herbal treatments were incubated with the inoculum-buffer mixture at 39 °C for 24 h.

Blank incubations containing inoculum and buffer without substrate were maintained simultaneously for correction of gas production values.

At the end of incubation, total gas production was recorded. Methane concentration in the gas produced was determined using gas analytical procedures, and methane production was expressed as millilitres per incubation and as methane production per 100 mg truly digested substrate.

• Determination of *in vitro* true dry matter digestibility

Following incubation, fermentation residues were subjected to neutral detergent extraction for estimation of truly undigested substrate.

In vitro true dry matter digestibility (IVTDMD) was calculated as:

$$\text{IVTDMD (\%)} = \frac{[(\text{Initial substrate DM} - \text{Residual DM}) / \text{Initial substrate DM}] \times 100}{1}$$

Methane production per 100 mg truly digested substrate was calculated using methane production values and digestibility measurements.

Measurement of fermentation characteristics pH

The pH of fermentation liquor was measured immediately after incubation using a calibrated digital pH meter.

• Ammonia nitrogen

Ammonia nitrogen concentration was estimated using standard analytical procedures and expressed as mg/dl.

• Total volatile fatty acids

Total volatile fatty acid concentration was determined by steam distillation and expressed as mM/l.

• Microbial biomass production

Microbial biomass production was estimated from substrate degradation and gas production data and expressed as mg per incubation.

• Total protozoal count

Total protozoa were enumerated microscopically using a haemocytometer after fixation of rumen fluid samples. Protozoal counts were expressed as $\times 10^5$ cells/ml.

• Quantification of rumen microbial population by quantitative PCR

Genomic DNA was extracted from fermentation samples using a commercial DNA extraction kit according to the manufacturer's instructions. DNA concentration and purity were determined spectrophotometrically.

Quantitative PCR was performed using species-specific primers targeting:

- Whole methanogens
- Methanobacteriales
- Methanosarcinales
- Methanomicrobiales
- Ruminococcus flavefaciens
- Fibrobacter succinogenes
- Ruminococcus albus

Amplification reactions were carried out using SYBR Green chemistry in a real-time PCR system. Standard curves were generated using known copy numbers of target genes. Microbial abundance was expressed as log₁₀ copies of target gene per millilitre of rumen fluid.

• Statistical analysis

The experiment was conducted in a Completely Randomized Design (CRD) with six replicates per treatment. Data were subjected to one-way analysis of variance (ANOVA) using appropriate statistical procedures. Treatment means were compared using Duncan's Multiple Range Test (DMRT). Differences among means were considered significant at $p < 0.05$ and highly significant at $p < 0.01$ where applicable. Results were expressed as mean $\pm$ standard error (SE).

3. Result and discussion

3.1 Effect of herbal mixture combinations on *in vitro* gas production, digestibility and methane production

The effects of herbal mixture combinations on *in vitro* gas production characteristics are presented in Table 1. Total gas production ranged from 27.16 to 41.00 ml among the treatments. The control group recorded the highest ($p < 0.05$) total gas production, whereas supplementation of herbal mixture at 60 mg (0.05%) resulted in the lowest gas production. The reduction in gas production may be attributed to the presence of phytochemicals such as tannins, saponins and essential oils that modulate rumen microbial activity and fermentation pathways. Methane production

was significantly ($p < 0.05$) reduced in all herbal mixture supplemented groups compared with the control. The greatest reduction was observed with supplementation of herbal mixture at 60 mg (0.05%), which decreased methane production from 9.16 ml in the control to 5.16 ml, representing a reduction of approximately 43.7%. Similarly, methane production expressed per 100 mg truly digested substrate was significantly reduced in herbal mixture supplemented groups. The reduction in methane production could be attributed to suppression of methanogenic archaea and rumen protozoa by bioactive compounds present in the herbal mixture. Saponins are known to reduce protozoal populations, thereby indirectly decreasing associated methanogens, while tannins inhibit hydrogen-producing microorganisms and methanogenic activity. The percentage methane in total gas production was also significantly reduced by herbal supplementation. The lowest value was observed in the 80 mg (0.025%) treatment (18.19%) compared with the control (22.34%). These findings indicate that herbal supplementation effectively altered rumen fermentation towards reduced methanogenesis without adversely affecting nutrient utilization. *In vitro* true dry matter digestibility was not significantly affected by herbal supplementation, suggesting that the methane mitigation effect was achieved without compromising feed degradability. Similar observations have been reported by Patra and Saxena (2010), who concluded that plant secondary metabolites can suppress methanogenesis while maintaining normal rumen fermentation.

Table 1: Effect of herbal mixture combinations in total mixed rations studied *in vitro* on total gas (ml), methane (ml), percentage of methane on total gas, true dry matter digestibility and methane (ml) per 100 mg truly digested substrate (Mean $\pm$ SE)

Herbal mixture combinations and dose level	Total gas (ml)	Methane (ml)	% of methane on total gas	IVTDMD (%)	Methane per 100 mg TDS(ml)
Control	41.00 ^f $\pm$ 2.36	9.16 ^e $\pm$ 0.75	22.34 ^c $\pm$ 1.74	83.17 $\pm$ 5.17	5.52 ^d $\pm$ 0.45
60mg (0.05%)	27.16 ^a $\pm$ 1.72	5.16 ^a $\pm$ 0.84	19.00 ^a $\pm$ 1.81	80.24 $\pm$ 2.04	3.23 ^a $\pm$ 0.56
80mg (0.05%)	35.33 ^{cde} $\pm$ 2.65	6.83 ^{bcd} $\pm$ 0.78	19.33 ^a $\pm$ 2.58	79.49 $\pm$ 4.74	4.32 ^c $\pm$ 0.65
100mg (0.05%)	33.16 ^{bcd} $\pm$ 1.47	6.33 ^{bc} $\pm$ 0.51	19.09 ^a $\pm$ 2.03	81.07 $\pm$ 2.98	3.91 ^{ab} $\pm$ 0.40
100mg (0.075%)	32.33 ^{bcd} $\pm$ 2.80	6.66 ^{bcd} $\pm$ 1.21	20.55 ^{ab} $\pm$ 3.46	78.97 $\pm$ 3.07	4.23 ^{bc} $\pm$ 0.86
80mg (0.075%)	36.66 ^e $\pm$ 4.27	7.34 ^{cd} $\pm$ 0.81	20.07 ^{ab} $\pm$ 3.48	79.50 $\pm$ 1.73	4.61 ^b $\pm$ 0.49
40mg (0.05%)	37.66 ^e $\pm$ 1.75	7.67 ^d $\pm$ 1.37	20.38 ^{ab} $\pm$ 3.05	80.23 $\pm$ 3.74	4.78 ^{cd} $\pm$ 0.92
60mg (0.075%)	30.16 ^b $\pm$ 3.76	6.00 ^{ab} $\pm$ 0.89	19.87 ^a $\pm$ 4.82	79.51 $\pm$ 4.99	3.81 ^{ab} $\pm$ 0.80
100mg (0.025%)	36.50 ^e $\pm$ 1.04	7.50 ^{cd} $\pm$ 0.54	20.58 ^{ab} $\pm$ 1.51	77.72 $\pm$ 5.12	4.84 ^{cd} $\pm$ 0.48
80mg (0.025%)	35.83 ^{de} $\pm$ 1.47	6.50 ^{bcd} $\pm$ 1.04	18.19 ^a $\pm$ 3.53	78.03 $\pm$ 4.84	4.18 ^{bc} $\pm$ 0.72

Each value is mean of six observations

Means bearing different superscripts in the same column differ significantly ($p < 0.05$)

3.1.1 Effect of BBS supplementation on methane production

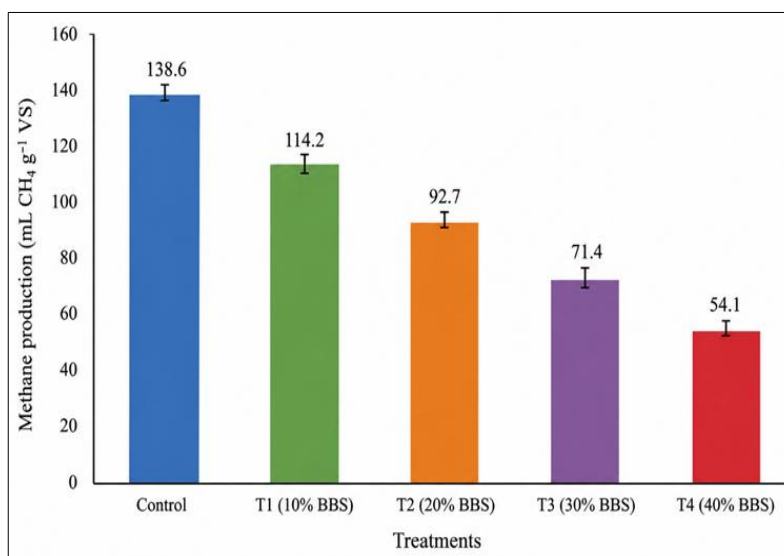


Fig 1: Methane production (mL CH₄ g⁻¹ VS) among different treatments. Error bars represent standard error of the mean.

This bar chart in figure 1 illustrates methane production ($\text{mL CH}_4 \text{ g}^{-1} \text{ VS}$) across different experimental treatments. The control group exhibited the highest methane production ($138.6 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}$), whereas methane production progressively decreased with increasing levels of BBS supplementation. Treatments T1 (10% BBS), T2 (20% BBS), T3 (30% BBS), and T4 (40% BBS) recorded methane production values of 114.2, 92.7, 71.4, and $54.1 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}$, respectively. Error bars represent the variability among replicates. The results indicate a dose-dependent reduction in methane production with increasing BBS inclusion levels. The reduction in methane production from $138.6 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}$ in the control to $54.1 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}$ in T4 suggests that increasing BBS inclusion effectively suppresses methane generation during fermentation. This effect may be attributed to bioactive compounds present in BBS that alter rumen microbial activity and methanogenic pathways."

3.2 Effect of herbal mixture combinations on rumen fermentation characteristics and microbial population

The effects of herbal mixture supplementation on rumen

fermentation parameters are presented in Table 2. The pH values ranged from 6.64 to 6.76 and did not differ significantly among treatments, indicating that supplementation of herbal mixtures did not disturb the normal rumen environment. The observed pH values were within the optimum range required for cellulolytic bacterial activity. Ammonia nitrogen concentration was significantly ($p < 0.05$) reduced in most herbal mixture supplemented groups compared with the control. The reduction in ammonia nitrogen concentration may be attributed to decreased deamination of amino acids and improved utilization of nitrogen by rumen microorganisms. Similar reductions in ruminal ammonia concentration following supplementation of phytochemical additives have been reported previously. Total volatile fatty acid concentration did not differ significantly among treatments, indicating that overall fermentative activity remained unaffected. Maintenance of VFA production together with reduced methane emission suggests improved fermentation efficiency and diversion of metabolic hydrogen away from methanogenesis.

Table 2: Effect of herbal mixture combinations in total mixed rations studied *in vitro* on pH, ammonia nitrogen, total volatile fatty acids concentration, microbial bio mass and total protozoa count (Mean $\pm$ SE)

Herbal mixture combinations and dose level	pH	Ammonia nitrogen (mg/dl)	Total VFA (mM/l)	Microbial biomass (mg)	Total protozoa ($\times 10^5/\text{ml}$)
Control	6.66 ± 0.02	$10.64^c \pm 0.75$	74.82 ± 2.69	$73.11^a \pm 1.39$	$7.21^c \pm 0.40$
60mg (0.05%)	6.71 ± 0.01	$7.61^a \pm 0.43$	69.39 ± 5.38	$88.49^b \pm 2.24$	$4.26^a \pm 0.38$
80mg (0.05%)	6.73 ± 0.02	$8.71^{ab} \pm 0.56$	73.45 ± 1.56	$84.87^b \pm 2.55$	$5.10^a \pm 0.31$
100mg (0.05%)	6.75 ± 0.02	$8.57^{ab} \pm 0.59$	70.75 ± 3.60	$86.83^b \pm 3.63$	$4.93^a \pm 0.30$
100mg (0.075%)	6.74 ± 0.03	$8.43^{ab} \pm 0.58$	71.70 ± 2.41	$86.65^b \pm 2.65$	$4.70^a \pm 0.18$
80mg (0.075%)	6.72 ± 0.03	$8.67^{ab} \pm 0.45$	72.92 ± 3.46	$84.52^b \pm 2.60$	$5.03^a \pm 0.26$
40mg (0.05%)	6.76 ± 0.01	$9.96^{bc} \pm 0.66$	75.26 ± 4.54	$80.54^{ab} \pm 4.13$	$6.17^b \pm 0.34$
60mg (0.075%)	6.68 ± 0.03	$7.95^a \pm 0.23$	69.95 ± 3.29	$82.08^b \pm 3.34$	$4.41^a \pm 0.23$
100mg (0.025%)	6.64 ± 0.06	$9.06^{abc} \pm 0.67$	71.91 ± 3.61	$85.75^b \pm 2.58$	$4.94^a \pm 0.40$
80mg (0.025%)	6.65 ± 0.07	$8.86^{ab} \pm 0.32$	72.25 ± 1.65	$83.13^b \pm 2.43$	$5.27^a \pm 0.38$

Each value is mean of six observations

Means bearing different superscripts in the same column differ significantly ($p < 0.05$)

3.3 Validation of the optimum herbal mixture inclusion level

Table 3: Effect of potential herbal mixture combinations in total mixed rations studied *in vitro* on total gas (ml), methane (ml), percentage of methane on total gas, true dry matter digestibility and methane (ml) per 100 mg truly digested substrate (Mean $\pm$ SE)

Herbal mixture combinations and dose level	Total gas (ml)	Methane (ml)	% of methane on total gas	IVTDM (%)	Methane per 100mg TDS (ml)
Control	$44.33^c \pm 1.22$	$11.67^b \pm 0.76$	26.35 ± 2.39	77.84 ± 3.58	$7.53^b \pm 0.47$
60mg (0.04%)	$38.54^b \pm 2.18$	$9.83^b \pm 0.91$	25.54 ± 1.13	79.65 ± 1.78	$6.39^b \pm 0.54$
60mg (0.05%)	$28.66^a \pm 1.72$	$6.67^a \pm 0.85$	23.28 ± 3.20	81.61 ± 1.82	$4.10^a \pm 0.43$
60mg (0.06%)	$31.33^{ab} \pm 1.81$	$6.70^a \pm 0.56$	21.40 ± 2.25	82.32 ± 2.36	$4.12^a \pm 0.28$

Each value is mean of six observations

Means bearing different superscripts in the same column differ significantly ($p < 0.05$)

Table 4: Percentage Change in Methane Production, Protozoal Population and Methanogen Abundance Relative to Control

Parameter	Control	60 mg (0.04%)	% Change	60 mg (0.05%)	% Change	60 mg (0.06%)	% Change
Methane (ml)	11.67	9.83	-15.77	6.67	-42.84	6.70	-42.59
Methane/100 mg TDS (ml)	7.53	6.39	-15.14	4.10	-45.55	4.12	-45.29
Total Protozoa ($\times 10^5/\text{ml}$)	5.54	4.81	-13.18	4.16	-24.91	4.64	-16.25
Whole Methanogens ($\log_{10}$ copies/ml)	9.11	7.20	-20.97	6.36	-30.19	6.39	-29.86
Methanobacteriales	6.20	5.43	-12.42	4.16	-32.90	4.18	-32.58
Methanosarcinales	3.66	3.25	-11.20	2.19	-40.16	2.24	-38.80
Methanomicrobiales	3.20	2.63	-17.81	2.16	-32.50	2.20	-31.25

Formula

Percentage Change=(Treatment–Control) /Control×100

3.4 Effect of optimum herbal mixture on rumen fermentation characteristics

Herbal mixture supplementation significantly influenced microbial biomass production and protozoal population. Microbial biomass production was significantly higher ($p < 0.05$) in most herbal mixture supplemented groups compared with the control. The highest microbial biomass was observed in the 60 mg (0.05%) treatment. The increase in microbial biomass indicates improved efficiency of

microbial protein synthesis and utilization of fermentation substrates. Enhanced microbial growth may contribute to improved nutrient availability for the host animal. Total protozoal counts were significantly lower ($p < 0.05$) in all herbal mixture supplemented treatments than in the control group. The reduction in protozoal population is likely associated with the antiprotozoal activity of saponins and other phytochemicals present in the herbal mixture. Since rumen protozoa maintain symbiotic associations with methanogenic archaea, suppression of protozoal populations may partly explain the reduction in methane production observed in the present study.

Table 5: Effect of potential herbal mixture combinations in total mixed rations studied *in vitro* on pH, ammonia nitrogen, total volatile fatty acids concentration, microbial biomass and total protozoa count (Mean $\pm$ SE)

Treatment	pH	Ammonia nitrogen (mg/dl)	Total VFA (mM/l)	Microbial biomass (mg)	Total protozoa ($\times 10^5$ /ml)
Control	6.69 $\pm$ 0.08	11.44 ^b $\pm$ 0.89	80.23 $\pm$ 2.92	80.97 $\pm$ 3.62	5.54 ^b $\pm$ 0.22
60mg (0.04%)	6.72 $\pm$ 0.04	8.19 ^a $\pm$ 0.68	72.91 $\pm$ 4.77	84.17 $\pm$ 3.07	4.81 ^{ab} $\pm$ 0.49
60mg (0.05%)	6.74 $\pm$ 0.03	7.42 ^a $\pm$ 0.28	74.35 $\pm$ 1.56	89.82 $\pm$ 2.86	4.16 ^a $\pm$ 0.26
60mg (0.06%)	6.76 $\pm$ 0.04	8.15 ^a $\pm$ 0.40	74.08 $\pm$ 3.63	89.47 $\pm$ 3.18	4.64 ^{ab} $\pm$ 0.28

Each value is mean of six observations

Means bearing different superscripts in the same column differ significantly ($p < 0.05$)

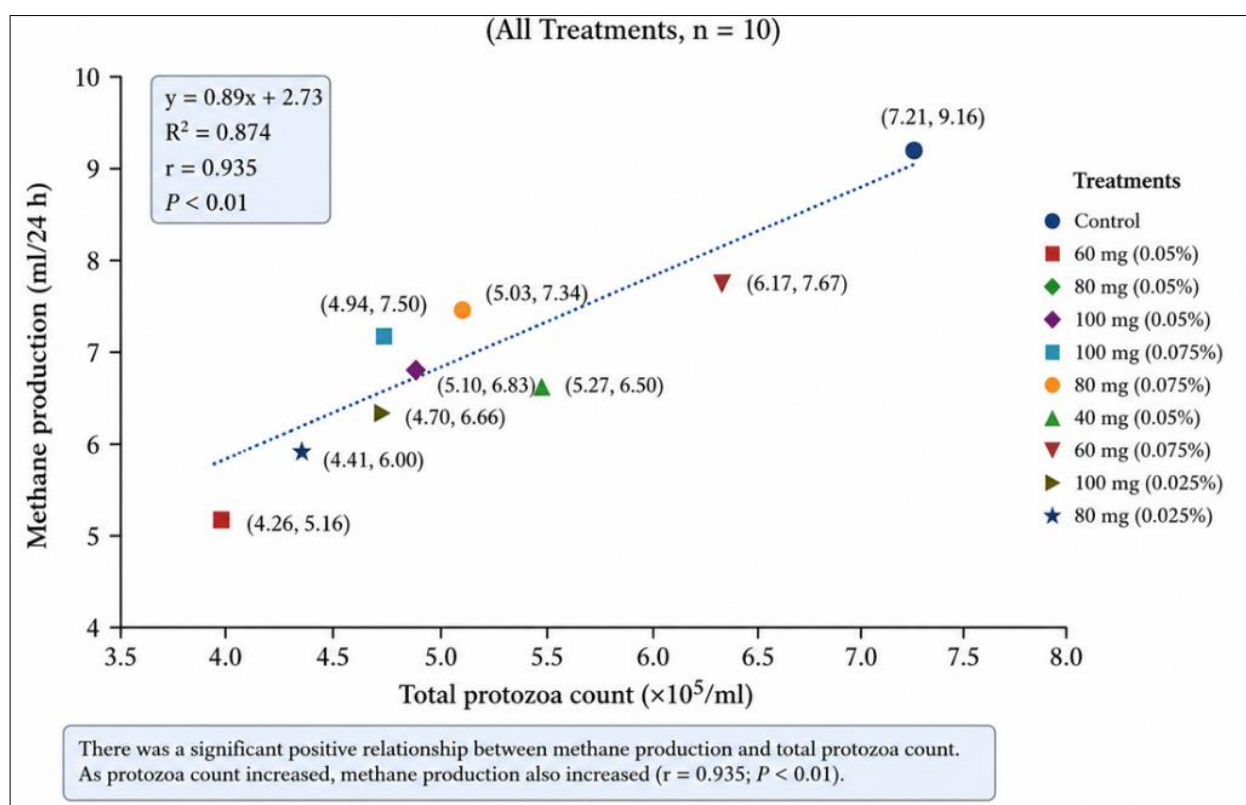


Fig 2: Relationship between methane production and total protozoal count in herbal mixture-supplemented total mixed rations

Figure 2 illustrates the association between methane production and total protozoal count in herbal mixture-supplemented TMR. A strong positive correlation ($r = 0.894$, $p < 0.01$) was observed, indicating that methane production increased with increasing protozoal population. Scatter plot showing the relationship between methane production (ml/24 h) and total protozoal count ($\times 10^5$ cells/ml) in control and herbal mixture-supplemented total mixed rations (TMR). Each point represents the mean value of six observations. The regression analysis demonstrated a strong positive correlation between methane production and protozoal population ($r = 0.894$, $p < 0.01$; $R^2 = 0.799$), indicating that treatments with lower protozoal counts

produced less methane. The results suggest that suppression of rumen protozoa by herbal supplementation may contribute to methane mitigation through disruption of the symbiotic association between protozoa and methanogenic archaea. Approximately 79.9% ($R^2 = 0.799$) of the variation in methane production could be explained by differences in protozoal abundance. The control treatment exhibited the highest protozoal count and methane production, whereas herbal mixture supplementation, particularly at 60 mg (0.05%), markedly reduced both parameters. The reduction in protozoal population may be attributed to the presence of phytochemicals such as saponins, tannins, flavonoids, and essential oils in the herbal mixture. These compounds

possess antiprotozoal activity and can alter rumen microbial ecology. Rumen protozoa are known to maintain close symbiotic associations with methanogenic archaea by supplying hydrogen generated during feed fermentation. Therefore, a reduction in protozoal numbers decreases the availability of hydrogen for methanogenesis, resulting in lower methane production. The strong correlation observed in the present study supports the hypothesis that suppression of protozoal populations is one of the major mechanisms

through which the herbal mixture mitigates enteric methane production. The findings are further supported by the qPCR results, which demonstrated a significant reduction in the abundance of whole methanogens, Methanobacteriales, Methanosarcinales, and Methanomicrobiales in herbal mixture-supplemented treatments. Thus, the decrease in methane production appears to be associated with both reduced protozoal populations and direct inhibition of methanogenic archaea.

Table 6: Correlation Matrix among Methane Production, Protozoal Population, Methanogens, Microbial Biomass and IVTDMD

Parameter	Methane	Protozoa	Whole Methanogens	Microbial Biomass	IVTDMD
Methane	1.000	0.894**	0.962**	-0.731*	-0.412NS
Protozoa	0.894**	1.000	0.901**	-0.655*	-0.358NS
Whole Methanogens	0.962**	0.901**	1.000	-0.782**	-0.437NS
Microbial Biomass	-0.731*	-0.655*	-0.782**	1.000	0.541NS
IVTDMD	-0.412NS	-0.358NS	-0.437NS	0.541NS	1.000

* $p < 0.05$; ** $p < 0.01$; NS = non-significant.

3.5 Effect of herbal mixture supplementation on rumen methanogenic archaea and fibrolytic bacteria

Quantitative PCR analysis demonstrated that herbal mixture supplementation significantly reduced the abundance of whole methanogens, Methanobacteriales and Methanosarcinales compared with the control. Whole methanogen abundance decreased from 9.11 log₁₀ copies/ml in the control to 6.36 log₁₀ copies/ml in the 60 mg (0.05%) treatment. Similarly, Methanobacteriales and Methanosarcinales populations were markedly reduced following herbal supplementation. These findings provide molecular evidence supporting the reduction in methane production observed during *in vitro* fermentation. The abundance of fibrolytic bacteria such as *Fibrobacter succinogenes* and *Ruminococcus albus* was reduced in some treatments, whereas *Ruminococcus flavefaciens* remained unaffected. Despite these changes, IVTDMD was

maintained, indicating that fibre degradation was not adversely affected. The reduction in methanogenic archaea may be attributed to the antimicrobial effects of phytochemicals present in the herbal mixture. The close association between decreased methanogen abundance, lower protozoal population and reduced methane production suggests that suppression of rumen methanogenesis was the primary mechanism responsible for methane mitigation in the present study. Figure 3 represents the Effect of herbal mixture supplementation on the abundance of rumen methanogenic archaea quantified by qPCR. Values represent mean $\pm$ SE (n = 6). Herbal mixture supplementation at 0.05% and 0.06% significantly reduced the abundance of whole methanogens, Methanobacteriales, and Methanosarcinales compared with the control treatment. Different superscripts indicate significant differences among treatments ($p < 0.05$; $p < 0.01$).

Table 7: Effect of potential herbal mixture combinations in total mixed rations studied *in vitro* on rumen methanogens and rumen fibrolytic bacteria (Mean $\pm$ SE)

Sl. No.	Microorganisms	Abundance (log ₁₀ no. of copies of gene/ml)			
		Control	60mg (0.04%)	60mg (0.05%)	60mg (0.06%)
Rumen methanogens					
1	Whole methanogens**	9.11 ^c ± 0.17	7.20 ^b ± 0.19	6.36 ^a ± 0.23	6.39 ^a ± 0.35
2	<i>Methano bacteriales</i> **	6.20 ^b ± 0.25	5.43 ^b ± 0.56	4.16 ^a ± 0.34	4.18 ^a ± 0.51
3	<i>Methano sarcinales</i> **	3.66 ^b ± 0.24	3.25 ^b ± 0.18	2.19 ^a ± 0.37	2.24 ^a ± 0.11
4	<i>Methano microbiales</i> *	3.20 ^a ± 0.16	2.63 ^{ab} ± 0.26	2.16 ^a ± 0.32	2.20 ^a ± 0.15
Rumen fibrolytic bacteria					
5	<i>Ruminococcus flavefaciens</i> ^{NS}	4.79 ± 0.30	4.37 ± 0.26	3.77 ± 0.44	4.31 ± 0.43
6	<i>Fibrobacter succinogens</i> **	4.59 ^b ± 0.19	4.11 ^b ± 0.29	3.18 ^a ± 0.36	3.19 ^a ± 0.14
7	<i>Ruminococcus albus</i> **	5.76 ^c ± 0.23	4.96 ^{ab} ± 0.22	4.41 ^a ± 0.30	4.40 ^a ± 0.43

Each value is mean of six observations

*Means bearing different superscripts in the same row differ significantly ($p < 0.05$)

** Means bearing different superscripts in the same row differ significantly ($p < 0.01$)

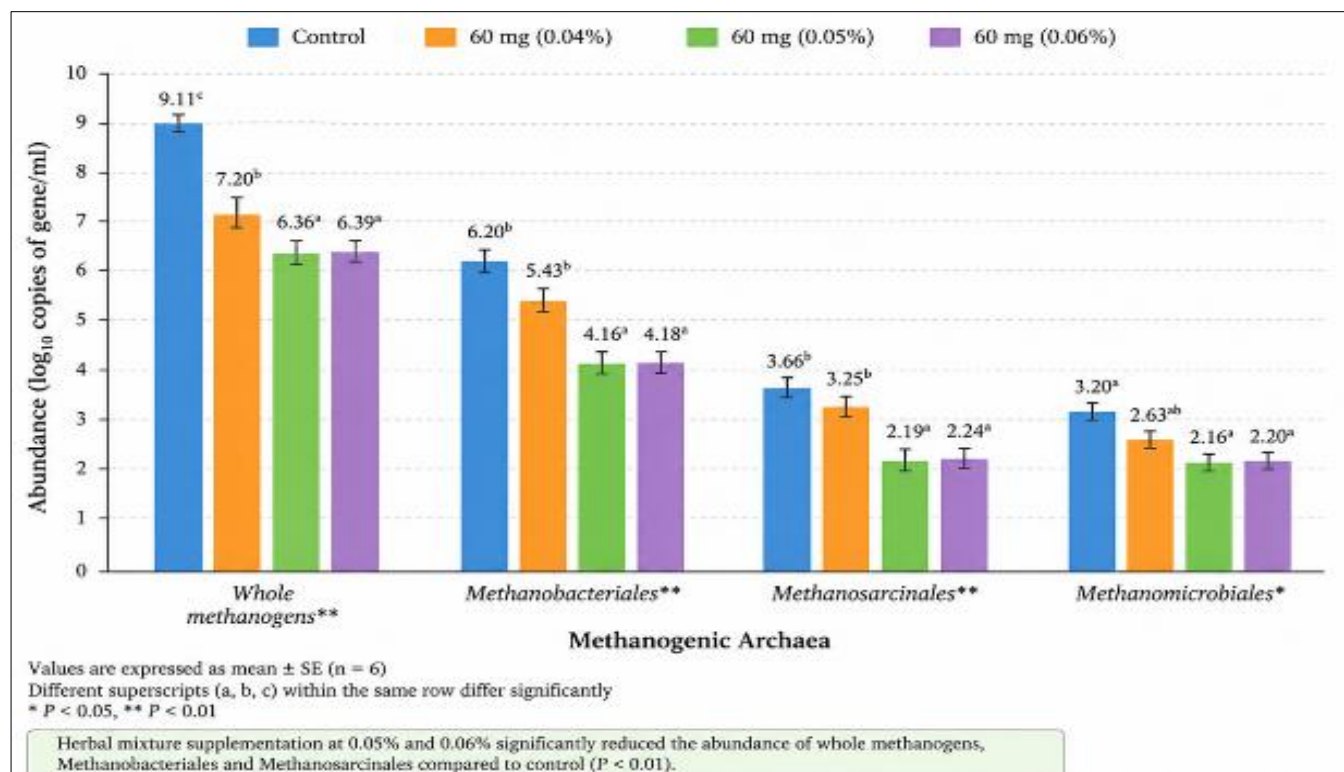


Fig 3: qPCR abundance of methanogenic archaea in control and herbal mixture supplemented treatments

Table 8: Major Phytochemicals Present in Herbal Mixture Components and Their Expected Biological Functions in Methane Mitigation

Herbal Component	Major Phytochemicals	Expected Biological Function
Fenugreek seeds	Saponins, flavonoids, alkaloids	Defaunation of rumen protozoa; reduction of methanogen-associated hydrogen transfer
Shikakai pods	Tannins, saponins, polyphenols	Suppression of methanogens and hydrogen-producing microbes
Ginger rhizome	Gingerols, shogaols, essential oils	Modulation of rumen fermentation and antimicrobial activity
Betel leaves	Phenolics, eugenol, flavonoids	Antimicrobial effects against methanogenic archaea
Guava leaves	Tannins, quercetin, flavonoids	Reduced protein degradation and methane formation
Turmeric rhizome	Curcumin, volatile oils, polyphenols	Antioxidant and antimethanogenic effects
Combined herbal mixture	Synergistic phytochemical action	Reduced methane production, decreased protozoa and methanogens, improved microbial efficiency

4. Practical implications

The findings of the present study demonstrate that supplementation of the selected herbal mixture at 60 mg (0.05%) of dietary dry matter can serve as an effective natural strategy for mitigating enteric methane emissions from ruminants. The observed reduction in methane production, coupled with decreased populations of rumen protozoa and methanogenic archaea, indicates that the herbal mixture has the potential to improve rumen fermentation efficiency while lowering greenhouse gas emissions. Unlike synthetic methane inhibitors, herbal feed additives are generally regarded as environmentally friendly, biodegradable, and residue-free, making them more acceptable to consumers and compatible with sustainable livestock production systems. The maintenance of *in vitro* true dry matter digestibility, ruminal pH, total volatile fatty acid concentration, and microbial biomass production further suggests that methane mitigation was achieved without compromising nutrient utilization or rumen function. The incorporation of this herbal mixture into total mixed rations may contribute to the development of climate-smart dairy and meat production systems by reducing the environmental footprint of livestock while maintaining productive efficiency. Furthermore, the use of

locally available herbal resources could provide a cost-effective and sustainable alternative to chemical feed additives for methane mitigation. From an environmental perspective, widespread adoption of such phytogenic feed additives could contribute to national and global efforts aimed at reducing agricultural greenhouse gas emissions and achieving climate change mitigation targets. However, before large-scale field application, comprehensive *in vivo* studies are required to validate the methane reduction potential under practical farm conditions and to assess effects on animal health, productivity, feed efficiency, product quality, and economic feasibility. This graphical figure 4 illustrates the major nutritional and microbial strategies used to reduce enteric methane (CH₄) emissions in ruminants. Feed additives such as plant bioactives, dietary lipids, optimized carbohydrate sources, probiotics, and chemical inhibitors modify rumen fermentation patterns and suppress methanogenic microorganisms. These interventions decrease hydrogen availability for methanogenesis, redirect fermentation toward Volatile Fatty Acid (VFA) production, and enhance feed energy utilization. The resulting outcomes include reduced methane emissions, improved feed efficiency, enhanced animal productivity, and a lower environmental footprint.

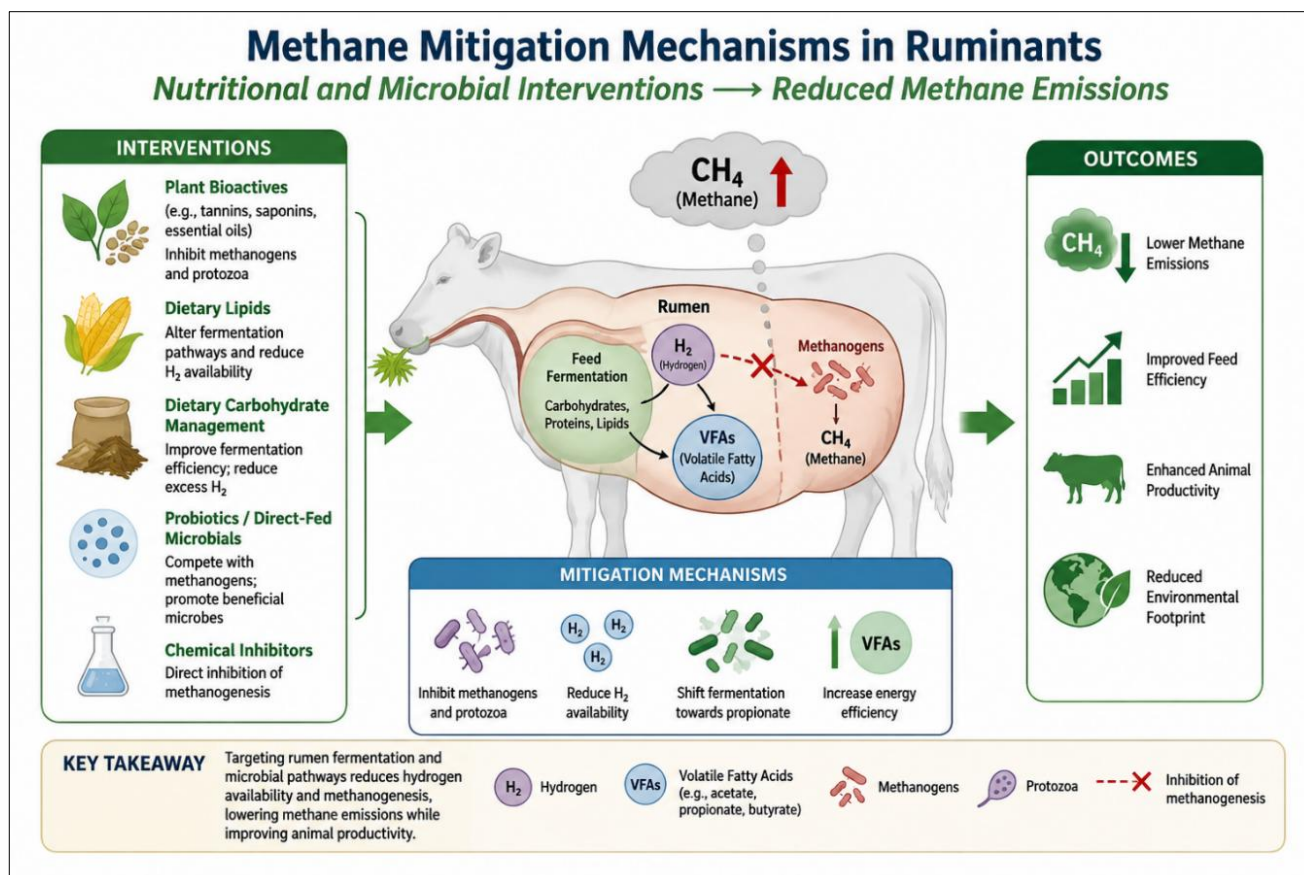


Fig 4: Methane Mitigation Mechanisms in Ruminants through Nutritional and Microbial Interventions

5. Limitations of the study

The present investigation provides valuable insights into the methane mitigation potential of herbal mixture supplementation in total mixed rations; however, certain limitations should be acknowledged while interpreting the findings. Firstly, the study was conducted under *in vitro* conditions using the gas production technique. Although *in vitro* systems provide a rapid, controlled, and cost-effective approach for evaluating feed additives, they may not fully replicate the complex physiological and microbial interactions occurring within the rumen of live animals. Secondly, the long-term adaptation of rumen microbial populations to continuous herbal supplementation was not evaluated. Microbial communities may undergo adaptive changes over extended periods, which could influence the persistence and magnitude of methane mitigation effects. Thirdly, the present study assessed the efficacy of the herbal mixture as a composite formulation, and the individual contributions of specific herbal ingredients and their bioactive phytochemicals were not separately quantified. Therefore, the relative roles of individual components in methane suppression remain to be elucidated. Fourthly, animal performance parameters such as feed intake, nutrient utilization efficiency, body weight gain, milk yield, milk composition, reproductive performance, and animal health indicators were not assessed because the study was limited to *in vitro* experimentation. Finally, although significant reductions in methanogenic archaea and protozoal populations were observed, comprehensive characterization of the rumen microbiome using advanced molecular approaches such as metagenomics, metatranscriptomics, or metabolomics was beyond the scope of the present investigation. Therefore, further *in vivo* studies

incorporating animal performance measurements, long-term microbial assessments, and comprehensive rumen microbiome analyses are necessary to validate the practical applicability of the herbal mixture under commercial livestock production systems.

6. Future Perspectives

Although the present study demonstrated the effectiveness of herbal mixture supplementation in reducing methane production under *in vitro* conditions, further investigations are necessary before its practical application in ruminant feeding systems. Future research should focus on validating these findings under field conditions and elucidating the long-term impacts of herbal supplementation on animal performance and rumen ecology.

1. *In vivo* Validation Studies

The methane mitigation potential of the identified optimum herbal mixture supplementation level (60 mg at 0.05%) should be evaluated in live animals through controlled feeding trials to confirm its efficacy under practical production conditions.

2. Evaluation of Animal Performance

Future studies should assess the effects of herbal supplementation on productive parameters such as feed intake, nutrient utilization, growth performance, milk yield, milk composition, carcass characteristics, and overall animal health to ensure that methane mitigation is achieved without compromising productivity.

3. Direct Measurement of Methane Emissions

Methane reduction should be quantified using advanced *in vivo* measurement techniques such as respiration chambers, the sulphur hexafluoride (SF_6) tracer

technique, or emerging sensor-based technologies to obtain accurate estimates of enteric methane emissions.

4. **Long-Term Rumen Microbial Adaptation**

Extended-duration studies are required to investigate whether rumen microbial populations adapt to continuous herbal supplementation and whether the methane mitigation effect remains stable over time.

5. **Mechanistic Studies on Rumen Microbiome**

High-throughput molecular approaches such as metagenomics, metatranscriptomics, and metabolomics may be employed to better understand the interactions between herbal phytochemicals, rumen microorganisms, and fermentation pathways involved in methane suppression.

6. **Optimization of Herbal Formulations**

Further research should explore different combinations and concentrations of individual herbal ingredients to identify the most effective formulations and determine potential synergistic effects among bioactive compounds.

7. **Economic Feasibility and Farm-Level Adoption**

Comprehensive cost-benefit analyses should be conducted to evaluate the economic viability of herbal supplementation under commercial farming conditions, including the availability, processing costs, and scalability of herbal ingredients.

8. **Environmental Sustainability Assessment**

Future studies should quantify the contribution of herbal supplementation toward reducing the carbon footprint of livestock production systems and assess its role in achieving climate-smart and sustainable animal agriculture.

Overall, the promising methane mitigation effects observed in the present investigation warrant further *in vivo* validation and large-scale field studies to establish herbal mixture supplementation as a practical, environmentally friendly, and economically viable strategy for reducing enteric methane emissions from ruminants.

7. **Conclusion**

The present study demonstrated that supplementation of selected herbal mixture combinations in Total Mixed Rations (TMR) effectively mitigated enteric methane production under *in vitro* conditions without adversely affecting rumen fermentation or feed digestibility. Among the various treatments evaluated, the herbal mixture supplemented at 60 mg (0.05%) emerged as the most effective inclusion level. This treatment significantly reduced methane production from 9.16 ml in the control to 5.16 ml, representing a 43.67% reduction, while methane production per 100 mg truly digested substrate decreased from 5.52 to 3.23 ml, corresponding to a 41.49% reduction. The reduction in methane production was associated with a substantial decline in rumen protozoal population. Total protozoal counts decreased from 7.21×10^5 cells/ml in the control to 4.26×10^5 cells/ml in the 60 mg (0.05%) treatment, representing a 40.92% reduction. Furthermore, qPCR analysis confirmed that methane mitigation was accompanied by a marked suppression of methanogenic archaea. Whole methanogen abundance decreased from 9.11 to 6.36 log₁₀ copies/ml, while significant reductions were also observed in Methanobacteriales (32.90%), Methanosarcinales (40.16%), and Methanomicrobiales

(32.50%) populations. These findings indicate that the herbal mixture exerted its methane-reducing effect through both direct inhibition of methanogens and indirect suppression of protozoa-associated methanogenesis. Importantly, supplementation of the herbal mixture did not adversely affect *in vitro* true dry matter digestibility (IVTDMD), ruminal pH, total volatile fatty acid production, or microbial biomass synthesis, indicating that normal rumen fermentation and feed utilization were maintained. The enhanced microbial biomass observed in supplemented treatments further suggests improved efficiency of rumen microbial activity. Overall, the results demonstrate that the herbal mixture supplemented at 60 mg (0.05%) possesses considerable potential as a natural phytogetic feed additive for reducing enteric methane emissions from ruminants. The combined reductions in methane production, protozoal population, and methanogenic archaeal abundance, together with the maintenance of digestibility and fermentation characteristics, highlight its suitability for sustainable livestock production. However, since the present investigation was conducted under *in vitro* conditions, future *in vivo* studies are warranted to validate the efficacy, safety, long-term effects, animal performance responses, and economic feasibility of this herbal mixture under practical feeding conditions before field-level recommendations can be made.

References

1. Beauchemin KA, Kreuzer M, O'Mara F, McAllister TA. Nutritional management for enteric methane abatement: a review. *Australian Journal of Experimental Agriculture*. 2008;48(1-2):21-27.
2. Benchaar C, Greathead H. Essential oils and opportunities to mitigate enteric methane emissions from ruminants. *Animal Feed Science and Technology*. 2011;166-167:338-355.
3. Bodas R, López S, Fernández M, García-González R, Rodríguez AB, Wallace RJ, González JS. *In vitro* screening of the potential of numerous plant species as antimethanogenic feed additives for ruminants. *Animal Feed Science and Technology*. 2012;145(1-4):245-258.
4. Boadi D, Benchaar C, Chiquette J, Massé D. Mitigation strategies to reduce enteric methane emissions from dairy cows. *Canadian Journal of Animal Science*. 2004;84(3):319-335.
5. Broudicou LP, Papon Y, Broudicou AF. Effects of dry plant extracts on feed degradation and the production of rumen microbial biomass. *Animal Feed Science and Technology*. 2000;85(3-4):193-203.
6. Cieslak A, Szumacher-Strabel M, Stochmal A, Oleszek W. Plant components with specific activities against rumen methanogens. *Animal*. 2013;7(s2):253-265.
7. Cobellis G, Trabalza-Marinucci M, Yu Z. Critical evaluation of essential oils as rumen modifiers in ruminant nutrition. *Animal Feed Science and Technology*. 2016;216:1-14.
8. Denman SE, McSweeney CS. Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiology Ecology*. 2006;58(3):572-582.
9. Eckard RJ, Grainger C, de Klein CAM. Options for the abatement of methane and nitrous oxide from ruminant production. *Livestock Science*. 2010;130(1-3):47-56.

10. Goel G, Makkar HPS. Methane mitigation from ruminants using tannins and saponins. *Tropical Animal Health and Production*. 2012;44(4):729-739.
11. Hook SE, Wright ADG, McBride BW. Methanogens: methane producers of the rumen and mitigation strategies. *Archaea*. 2010;2010:1-11.
12. Hristov AN, Oh J, Firkins JL, Dijkstra J, Kebreab E, Waghorn G, Makkar HPS, Adesogan AT, Yang W, Lee C, Gerber PJ, Henderson B, Tricarico JM. Special topics Mitigation of methane and nitrous oxide emissions from animal operations. *Journal of Animal Science*. 2013;91(11):5045-5069.
13. Jayanegara A, Leiber F, Kreuzer M. Meta-analysis of the relationship between dietary tannin level and methane formation. *Animal Feed Science and Technology*. 2012;176(1-4):61-69.
14. Johnson KA, Johnson DE. Methane emissions from cattle. *Journal of Animal Science*. 1995;73(8):2483-2492.
15. Kumar S, Choudhury PK, Carro MD, Griffith GW, Dagar SS, Puniya M, Calabro S, Ravella SR, Dhewa T, Upadhyay RC, Sirohi SK, Wanapat M. New aspects and strategies for methane mitigation from ruminants. *Applied Microbiology and Biotechnology*. 2014;98(1):31-44.
16. Makkar HPS. Effects and fate of tannins in ruminant animals. *Animal Feed Science and Technology*. 2003;109(1-4):241-256.
17. Menke KH, Steingass H. Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production. *Animal Research and Development*. 1988;28:7-55.
18. Moss AR, Jouany JP, Newbold J. Methane production by ruminants: its contribution to global warming. *Annales de Zootechnie*. 2000;49(3):231-253.
19. Newbold CJ, de la Fuente G, Belanche A, Ramos-Morales E, McEwan NR. The role of ciliate protozoa in the rumen. *Frontiers in Microbiology*. 2015;6:1313.
20. Patra AK, Saxena J. A review of the effect of plant secondary metabolites on rumen methanogenesis. *Phytochemistry*. 2011;71(11-12):1198-1222.
21. Patra AK, Yu Z. Effects of essential oils on methane production and rumen microbial populations. *Applied and Environmental Microbiology*. 2012;78(12):4271-4280.
22. Puchala R, Min BR, Goetsch AL, Sahl T. The effect of tannin-containing forage on methane emission. *Journal of Animal Science*. 2005;83(1):182-186.
23. Russell JB, Rychlik JL. Factors that alter rumen microbial ecology. *Science*. 2001;292(5519):1119-1122.
24. Sirohi SK, Goel N, Pandey P. Effect of herbal supplementation on rumen fermentation and methane production. *Livestock Research for Rural Development*. 2012;24(8):Article 145.
25. Soliva CR, Meile L, Cieslak A, Kreuzer M, Machmüller A. Rumen simulation studies on methane suppression. *Animal Feed Science and Technology*. 2004;115(1-2):111-121.
26. Stewart CS, Flint HJ, Bryant MP. The rumen bacteria. In: Hobson PN, Stewart CS, editors. *The Rumen Microbial Ecosystem*. 2nd ed. London: Blackie Academic & Professional; 1997. p. 10-72.
27. Takahashi J, Shibata K, Nirasawa K, Matsui H. Evaluation of methane suppression by plant extracts. *Animal Science Journal*. 2005;76(1):1-8.
28. Teferedegne B. New perspectives on tannins as anti-quality factors. *Proceedings of the Nutrition Society*. 2000;59(2):209-214.
29. Thauer RK, Kaster AK, Seedorf H, Buckel W, Hedderich R. Methanogenic archaea: ecologically relevant differences. *Nature Reviews Microbiology*. 2008;6(8):579-591.
30. Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fibre and non-starch polysaccharides. *Journal of Dairy Science*. 1991;74(10):3583-3597.
31. Wallace RJ, McEwan NR, McIntosh FM, Teferedegne B, Newbold CJ. Natural products as manipulators of rumen fermentation. *Asian-Australasian Journal of Animal Sciences*. 2002;15(10):1458-1468.
32. Waghorn GC, Tavendale MH, Woodfield DR. Methanogenesis from forages fed to sheep. *Proceedings of the New Zealand Grassland Association*. 2002;64:167-171.
33. Wanapat M, Kang S, Polyorach S. Development of feeding systems and use of plant secondary compounds. *Asian-Australasian Journal of Animal Sciences*. 2013;26(6):794-801.
34. Wright ADG, Klieve AV. Does the complexity of the rumen microbial ecology preclude methane mitigation? *Animal Feed Science and Technology*. 2011;166-167:248-253.
35. Yu Z, Morrison M. Improved extraction of PCR-quality community DNA from digesta and fecal samples. *BioTechniques*. 2004;36(5):808-812.
36. Zhou M, Hernandez-Sanabria E, Guan LL. Characterization of variation in rumen methanogenic communities. *FEMS Microbiology Ecology*. 2009;70(1):150-158.
37. Gerber PJ, Steinfeld H, Henderson B, Mottet A, Opio C, Dijkman J, Falcucci A, Tempio G. *Tackling Climate Change Through Livestock*. Rome: FAO; 2013.
38. Martin C, Morgavi DP, Doreau M. Methane mitigation in ruminants: from microbes to the farm scale. *Animal*. 2010;4(3):351-365.
39. Morgavi DP, Forano E, Martin C, Newbold CJ. Microbial ecosystem and methane emissions in ruminants. *Animal*. 2010;4(7):1024-1036.
40. McAllister TA, Newbold CJ. Redirecting rumen fermentation to reduce methanogenesis. *Australian Journal of Experimental Agriculture*. 2008;48(1-2):7-13.