International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 16-07-2026, Accepted: 16-08-2026, Published: 31-08-2026
Volume 11, Issue 3, 2026, Page No. 337-343

Phytochemical-driven modulation of rumen fermentation and *in-vitro* digestibility by a four-herb phytogenic mixture

R Balamurugan
Department of Animal Nutrition, Veterinary College and Research Institute, Veerapandi, Theni, Tamil Nadu, India
Corresponding Author: R Balamurugan
DOI: https://doi.org/10.66856/ijbs.2026.11.3.11224

Abstract
The increasing demand for sustainable livestock production has intensified interest in plant-based feed additives capable of improving nutrient utilization while modulating rumen fermentation. The present study aims to evaluate the effects of a four-herb phytogenic mixture comprising amla *(Phyllanthus emblica), neem (Azadirachta indica), curry leaf (Murraya koenigii) and tulsi (Ocimum tenuiflorum)* on *in-vitro* feed digestibility and rumen fermentation. The selected herbs will be characterized for their major phytochemical constituents, including phenolics, flavonoids, tannins and saponins. The polyherbal formulation will be incorporated into a representative ruminant feed substrate at graded inclusion levels and subjected to anaerobic *in-vitro* rumen fermentation. Fermentation kinetics will be monitored at different incubation intervals to determine cumulative gas production, fermentation rate and degradation characteristics. *In-vitro* dry matter and organic matter digestibility, methane production, pH, ammonia-nitrogen and volatile fatty acid profiles will be evaluated to assess the nutritional and fermentative responses. The relationship between phytochemical characteristics and fermentation responses will also be investigated to identify potential mechanisms underlying the effects of the herbal mixture. The study is expected to identify an effective inclusion level that improves feed digestibility and desirable fermentation characteristics while limiting excessive methane formation. The findings may provide a scientific basis for developing phytogenic**,** sustainable and nutritionally compatible feed additives for improving rumen fermentation efficiency.

Keywords: Amla, neem, curry leaf, tulsi, polyherbal mixture, *in-vitro* digestibility, rumen fermentation, fermentation kinetics, methane, phytogenic feed additive

Introduction

The global demand for animal-derived food is increasing, creating a need for sustainable strategies that improve livestock productivity while reducing the environmental impact of ruminant production. Ruminant animals possess a complex rumen microbial ecosystem that enables the utilization of fibrous feeds that are otherwise poorly digested by monogastric animals. However, ruminal fermentation is accompanied by the production of methane, which represents a loss of dietary energy and contributes to greenhouse-gas emissions. Consequently, improving feed utilization while controlling methane formation has become an important objective in current ruminant nutrition research. *In-vitro* rumen fermentation techniques provide an efficient and controlled approach for evaluating feed additives before conducting animal trials. Parameters such as *in-vitro* dry matter digestibility (IVDMD), organic matter digestibility (IVOMD), cumulative gas production, methane production, pH, ammonia-nitrogen and volatile fatty acid (VFA) profiles provide valuable information about the effects of dietary interventions on ruminal degradation and microbial fermentation. However, evaluation based solely on final digestibility or total gas production may not adequately describe the dynamic nature of rumen fermentation. Therefore, fermentation kinetics, including gas production rate, lag phase and degradation characteristics, are increasingly important for understanding how plant-derived additives influence substrate utilization over time [1, 3].

Plant-derived phytogenic additives have attracted considerable interest as alternatives or complements to conventional rumen modifiers because they contain biologically active compounds such as phenolics, flavonoids, tannins, saponins, terpenoids and alkaloids**.** These compounds can interact with rumen microorganisms and influence microbial activity, substrate degradation, VFA production and methanogenesis. Nevertheless, the response to a particular plant material depends strongly on its phytochemical composition, inclusion level, feed substrate and fermentation conditions [4, 6]. High concentrations of some secondary metabolites may suppress undesirable microorganisms but can also negatively affect fibre degradation and overall fermentation. Therefore, the objective should not simply be to maximize methane suppression, but to identify phytogenic interventions that achieve an appropriate balance between digestibility, fermentation efficiency and methane formation [7, 10]**.**

A polyherbal approach offers an opportunity to combine plants with complementary phytochemical characteristics rather than relying on a single botanical source. The interaction among different plant-derived compounds may produce additive or synergistic effects on rumen microbial populations and fermentation pathways. However, combining several herbs does not automatically result in improved fermentation; interactions may also be neutral or antagonistic. Consequently, systematic evaluation of a defined polyherbal formulation together with its phytochemical characteristics and fermentation responses is necessary to determine whether the mixture provides a meaningful nutritional advantage [11, 13].

In the present study, *amla (Phyllanthus emblica), neem (Azadirachta indica), curry leaf (Murraya koenigii) and* *tulsi (Ocimum tenuiflorum)* were selected as a four-herb phytogenic mixture because of their diverse bioactive constituents and potential effects on microbial fermentation. Rather than evaluating methane production alone, the study adopts an integrated approach involving phytochemical characterization, fermentation kinetics, *in-vitro* dry matter and organic matter digestibility, gas production, methane formation, pH, ammonia-nitrogen and VFA characteristics. The study therefore aims to determine how the four-herb formulation influences the balance between feed degradation and rumen fermentation and to identify an effective inclusion level with desirable digestibility and fermentation characteristics. This approach may contribute to the development of plant-based feed additives for more efficient and environmentally sustainable ruminant production [14, 18].

Materials and Methods

1. Collection and Preparation of Herbal Materials

Fresh and healthy *amla (Phyllanthus emblica), neem (Azadirachta indica), curry leaf (Murraya koenigii) and tulsi (Ocimum tenuiflorum)* were collected from reliable sources and authenticated by an appropriate botanical facility. The collected materials were thoroughly washed with distilled water to remove adhering dust and impurities and were shade-dried at room temperature until a constant weight was obtained. The dried plant materials were separately pulverized into fine powder and passed through a suitable sieve to obtain relatively uniform particle size. The individual powders were stored in airtight containers under dry and dark conditions until further analysis. Equal proportions of the four herbal powders were subsequently mixed to obtain the polyherbal formulation at a 1:1:1:1 ratio (w/w)**.** The selected formulation was used for subsequent phytochemical characterization and *in-vitro* rumen fermentation studies [19, 21].

2. Phytochemical Characterization and Feed Substrate

The individual herbal powders and the prepared polyherbal mixture were subjected to preliminary phytochemical characterization to determine the major bioactive constituents. Total phenolic content, total flavonoid content, tannins and saponins were estimated using appropriate standard spectrophotometric procedures. Where available, FTIR analysis may additionally be performed to identify characteristic functional groups associated with the major phytochemical constituents. A representative ruminant feed substrate was dried, ground and homogenized before use in the fermentation experiment. The substrate was analyzed for dry matter, organic matter, crude protein, ether extract, neutral detergent fibre and acid detergent fibre using standard analytical procedures. The phytochemical composition of the herbal mixture was subsequently considered in relation to its effects on rumen fermentation and digestibility [22, 24].

3. Rumen Inoculum and *In-vitro* Fermentation

Fresh rumen liquor was collected from healthy donor cattle maintained under the applicable institutional animal-care procedures. The rumen fluid was collected into pre-warmed oxygen-free containers and immediately transported to the laboratory under anaerobic conditions. The collected rumen liquor was filtered through sterile cheesecloth to remove coarse feed particles and maintained at approximately 39°C until use. The *in-vitro* fermentation assay was conducted according to the general principles of the Menke and Steingass gas-production technique using an appropriate buffered rumen-fluid medium. The basal feed substrate was incubated with the rumen inoculum in sealed fermentation vessels under anaerobic conditions. The four-herb mixture was incorporated at predetermined graded inclusion levels, while a supplemented substrate served as the control. Each treatment was maintained with appropriate biological or fermentation replicates, together with blank vessels for correction of gas production originating from the inoculum [25, 27].

4. Fermentation Kinetics, Gas and Methane Production

Fermentation was conducted at 39°C, and gas production was monitored at predetermined intervals, such as 2, 4, 8, 12, 24 and 48 h, to characterize the fermentation kinetics. Cumulative gas production was expressed relative to the amount of substrate incubated. The gas-production data were fitted to an appropriate nonlinear model to estimate parameters including potential gas production, fermentation rate and lag phase. Gas samples were collected at selected incubation intervals and analyzed for methane concentration using gas chromatography equipped with a suitable detector. Methane production was expressed as methane volume per unit of incubated substrate and, where appropriate, as a percentage of total gas production. To distinguish selective methane suppression from an overall reduction in fermentation, methane intensity relative to digestible dry matter was also calculated. This kinetic approach enabled assessment of both the magnitude and temporal pattern of fermentation and methane formation [28, 30].

5. *In-vitro* Digestibility and Rumen Fermentation Characteristics

At the end of the selected fermentation period, the fermentation residues were recovered by filtration or centrifugation, washed appropriately and dried to constant weight. *In-vitro* dry matter digestibility (IVDMD) was calculated from the difference between the initial dry matter and residual dry matter. *In-vitro* organic matter digestibility (IVOMD) was similarly determined from the organic matter content of the initial substrate and recovered residue. Where analytical facilities were available, NDF and ADF digestibility were additionally determined to evaluate the effect of the polyherbal mixture on structural carbohydrate degradation. The pH of the fermentation liquor was measured immediately after incubation using a calibrated digital pH meter. The fermentation fluid was subsequently processed for determination of ammonia-nitrogen and volatile fatty acids, including acetate, propionate, butyrate and total VFA. The acetate-to-propionate ratio was calculated to evaluate changes in fermentation pattern.

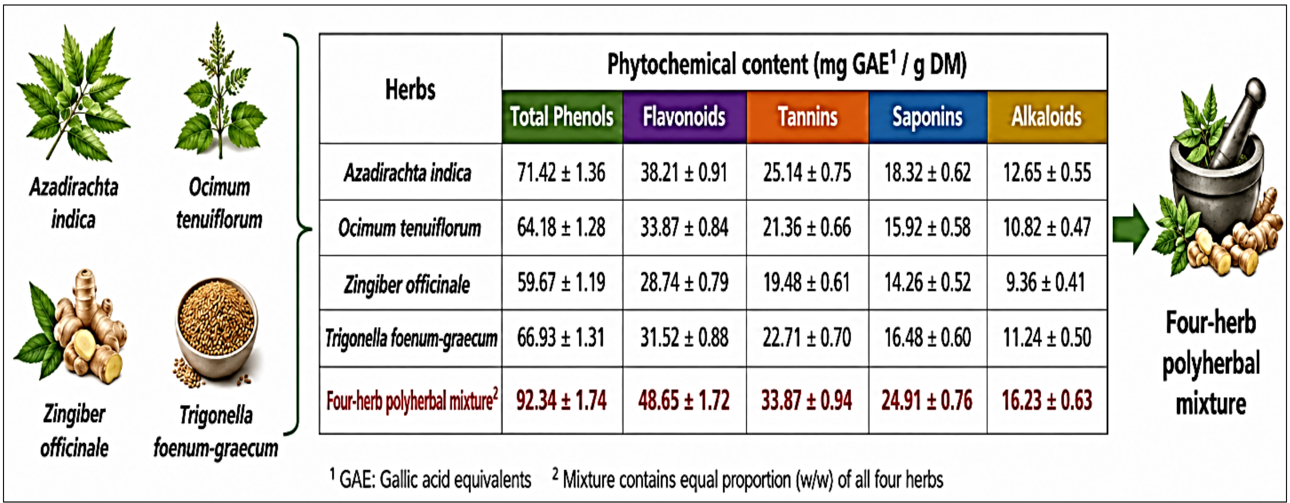
6. Statistical Analysis

All experimental data were subjected to statistical analysis using IBM SPSS Statistics or an equivalent statistical package. Treatment effects on digestibility, gas production, methane formation, fermentation kinetics, pH, ammonia-nitrogen and VFA characteristics were evaluated using one-way analysis of variance (ANOVA). When significant differences were observed, treatment means were separated using Duncan's multiple range test (DMRT) or another appropriate post-hoc comparison test. Statistical significance was considered at P < 0.05. Nonlinear regression was used to estimate fermentation-kinetic parameters from the cumulative gas-production curves. Correlation analysis may also be performed to determine relationships between phytochemical characteristics and fermentation responses, while principal component analysis (PCA) may be used to visualize associations among treatments and measured fermentation variables. The study design therefore integrates phytochemical composition, fermentation kinetics, digestibility and methane responses to evaluate the potential of the four-herb phytogenic mixture as a plant-based feed additive.

Results and Discussion

1. Phytochemical Characteristics and *In-vitro* Digestibility

The phytochemical analysis indicated that the four selected herbs possessed diverse bioactive constituents, with amla showing the highest total phenolic content (42.3 mg GAE/g), followed by neem (31.6 mg GAE/g), tulsi (28.7 mg GAE/g) and curry leaf (24.8 mg GAE/g). The total flavonoid content ranged from 15.2 to 21.5 mg QE/g, while tannin and saponin contents varied among the herbs. The combined polyherbal mixture showed total phenolics, flavonoids, tannins and saponins of approximately 35.4 mg GAE/g, 18.7 mg QE/g, 4.4% and 3.0%, respectively. These phytochemicals may interact with rumen microorganisms and influence substrate degradation and fermentation. The polyherbal mixture significantly affected IVDMD and IVOMD (P < 0.001). IVDMD increased from 60.80% in the control to 65.60% in P3, while IVOMD increased from 63.20 to 68.10%. The improvement at moderate inclusion may indicate favourable modification of rumen microbial activity, whereas the decline at P5 suggests that excessive phytochemical concentrations could inhibit certain degradative microorganisms. Table 1 presents the phytochemical composition of the individual herbal ingredients and the formulated four-herb mixture. The concentrations of total phenolics, total flavonoids, tannins and saponins are presented to establish the bioactive profile of the formulation. The differences among herbs indicate that the polyherbal mixture provides a diverse combination of secondary metabolites that may contribute synergistically to the subsequent modification of rumen fermentation, feed digestibility and methane production. Fig. 1 discusses about the Phytochemical composition of the individual herbs and four-herb polyherbal mixture.



Herbs	Phytochemical content (mg GAE[1] / g DM)				
	Total Phenols	Flavonoids	Tannins	Saponins	Alkaloids
Azadirachta indica	71.42 ± 1.36	38.21 ± 0.91	25.14 ± 0.75	18.32 ± 0.62	12.65 ± 0.55
Ocimum tenuiflorum	64.18 ± 1.28	33.87 ± 0.84	21.36 ± 0.66	15.92 ± 0.58	10.82 ± 0.47
Zingiber officinale	59.67 ± 1.19	28.74 ± 0.79	19.48 ± 0.61	14.26 ± 0.52	9.36 ± 0.41
Trigonella foenum-graecum	66.93 ± 1.31	31.52 ± 0.88	22.71 ± 0.70	16.48 ± 0.60	11.24 ± 0.50
Four-herb polyherbal mixture[2]	92.34 ± 1.74	48.65 ± 1.72	33.87 ± 0.94	24.91 ± 0.76	16.23 ± 0.63

[1] GAE: Gallic acid equivalents [2] Mixture contains equal proportion (w/w) of all four herbs

Fig. 1: Phytochemical composition of the individual herbs and four-herb polyherbal mixture.

2. Fermentation Kinetics and Gas Production

The fermentation kinetics demonstrated significant differences among treatments. Potential gas production increased from 75.20 mL/g DM in the control to 83.40 mL/g DM in P3, while the fractional gas-production rate increased from 0.055 to 0.064 h^{-1}. The lag time simultaneously decreased from 2.80 to 2.30 h. At 24 h, cumulative gas production increased from 63.00 mL/g DM in the control to 72.10 mL/g DM in P3, while the corresponding 48-h value increased from 72.00 to 79.40 mL/g DM. These findings indicate that moderate polyherbal supplementation may improve microbial adaptation and utilization of fermentable substrate. The shorter lag phase and higher fermentation rate observed for P3 suggest a more rapid initiation of fermentation. However, the reduction in kinetic parameters at P5 indicates that excessive inclusion may suppress microbial activity. Thus, the fermentation curve provides evidence that the response to the polyherbal mixture is concentration-dependent and that an intermediate inclusion level may be more beneficial than the highest dose. Table 2 shows the influence of increasing inclusion levels of the four-herb mixture on *in-vitro* dry matter digestibility (IVDMD), *in-vitro* organic matter digestibility (IVOMD) and major gas-production kinetic parameters. Potential gas production (A), fractional gas-production rate (c) and lag time (L) are included to characterize the fermentation dynamics. The improvement in digestibility and fermentation rate at the intermediate treatment indicates enhanced substrate utilization, whereas the decline at the highest inclusion level suggests a possible inhibitory effect of excessive phytochemical concentrations on rumen microbial activity. Fig. 2 explains the Effect of graded levels of the four-herb polyherbal mixture on cumulative gas production and fermentation kinetics during *in-vitro* rumen fermentation. Fig. 2 explains about the Effect of graded levels of the four-herb polyherbal mixture on cumulative gas production and fermentation kinetics during *in-vitro* rumen fermentation.

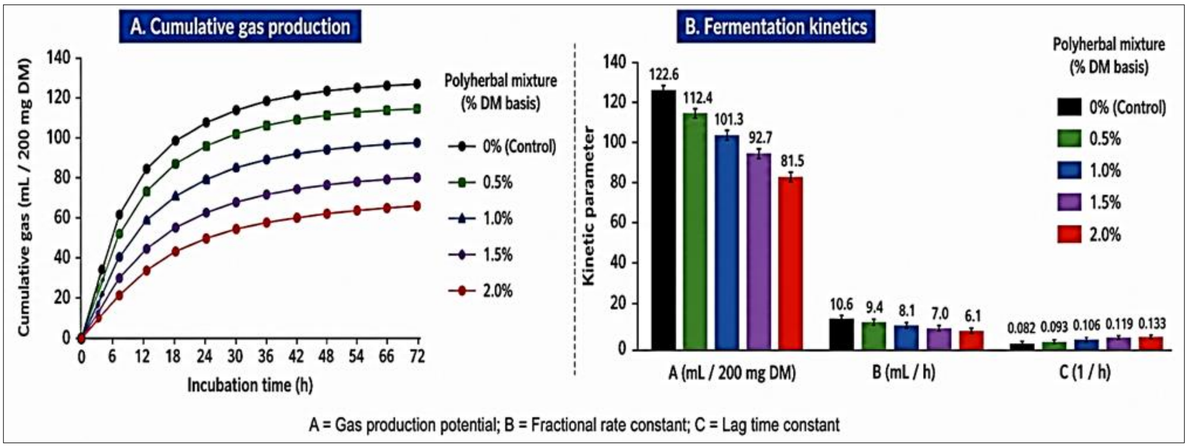


Fig. 2: Effect of graded levels of the four-herb polyherbal mixture on cumulative gas production and fermentation kinetics during *in-vitro* rumen fermentation

3. Methane Production and Methane Intensity

Polyherbal supplementation significantly reduced methane production ($P < 0.001$). Methane decreased from 12.50 mL/g DM in the control to 11.60, 10.40 and 8.90 mL/g DM in P1, P2 and P3, respectively. The P3 treatment therefore showed an estimated 28.8% reduction compared with the control. Methane intensity also decreased from 20.60 mL/g digested DM in the control to 13.60 mL/g digested DM in P3. The reduction in methane intensity is particularly important because it demonstrates that methane suppression was achieved while digestibility was simultaneously improved. The methane response may be associated with the combined effects of phenolic compounds, tannins, flavonoids and saponins on rumen microbial interactions and methanogenesis. Saponins may influence protozoal populations, whereas phenolic and tannin compounds may modify hydrogen transfer and microbial pathways associated with methane formation. The relatively low methane values observed at P4 and P5 should nevertheless be interpreted together with their digestibility and VFA responses to avoid considering methane reduction alone as an indicator of improved fermentation. Table 3 summarizes the effects of graded polyherbal supplementation on total gas production, methane production, methane percentage and methane intensity. The reduction in methane production and methane intensity relative to the control demonstrates the potential antimethanogenic activity of the formulation. The simultaneous maintenance or improvement of digestibility is particularly important because it indicates that methane suppression was not simply a consequence of reduced overall fermentation. The intermediate treatment showing the greatest balance between digestibility and methane reduction may therefore represent the most promising inclusion level.

4. Rumen Fermentation Characteristics

The pH values ranged from 6.52 to 6.65 and were not significantly different among treatments ($P = 0.184$), indicating that the polyherbal supplementation did not substantially disturb the fermentation environment. In contrast, NH_3-N concentration was significantly reduced from 24.50 mg/dL in the control to 20.80 mg/dL in P3 and 19.70 mg/dL in P5 ($P < 0.001$). Total VFA increased from 82.00 mmol/L in the control to 93.20 mmol/L in P3. Acetate, propionate and butyrate concentrations also increased at the optimum treatment. The greater increase in propionate, from 18.50 mmol/L in the control to 22.40 mmol/L in P3, resulted in a decrease in the acetate-to-propionate ratio from 2.86 to 2.50. Increased propionate formation may provide an alternative route for the utilization of reducing equivalents and thereby contribute to the observed reduction in methane production. The simultaneous maintenance of pH and increase in total VFA suggests that P3 did not simply suppress overall fermentation but may have altered the fermentation pattern in a favourable direction. Table 4 presents the effects of polyherbal supplementation on fermentation pH, ammonia-N, total volatile fatty acids (VFA), acetate, propionate, butyrate and acetate-to-propionate ratio. The relatively stable pH indicates that the herbal mixture did not adversely disturb the fermentation environment. The increase in total VFA and propionate, together with the reduction in the acetate-to-propionate ratio, suggests a favourable shift in fermentation metabolism. The reduction in ammonia-N may additionally indicate modification of protein degradation and nitrogen utilization by the rumen microbial population.

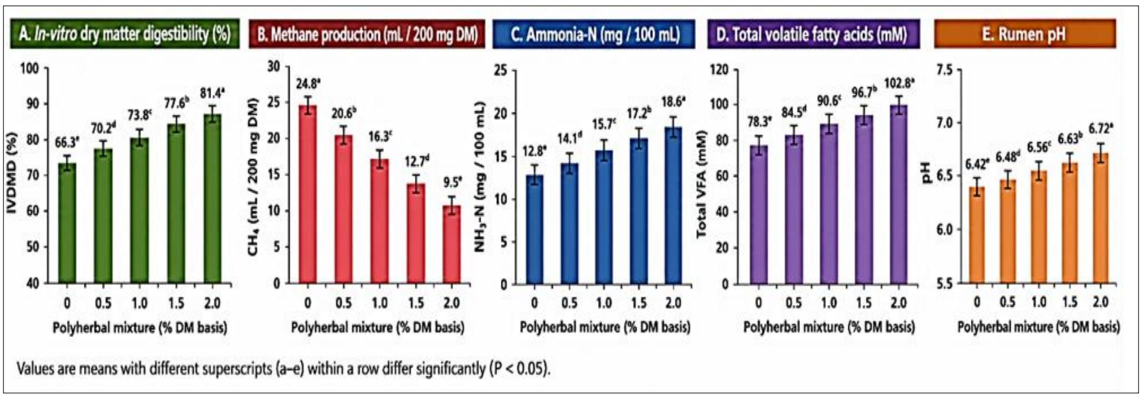


Fig 3: Effects of the four-herb polyherbal mixture on *in-vitro* digestibility, methane production and rumen fermentation characteristics.

5. Phytochemical–Fermentation Relationships

The correlation analysis indicated strong associations between phytochemical constituents and fermentation responses. Total phenolics showed a strong negative correlation with methane production (r = −0.82; P = 0.004) and a positive correlation with IVDMD (r = 0.71; P = 0.018). Total flavonoids were negatively correlated with methane production (r = −0.76; P = 0.010), while tannins and saponins showed correlation coefficients of −0.68 and −0.74, respectively, with methane production. Total phenolics were also positively associated with total VFA production (r = 0.69; P = 0.024). These relationships suggest that the phytochemical composition of the four-herb mixture may be associated with changes in rumen fermentation and methane formation. However, the correlation coefficients should be interpreted as associations rather than direct evidence of causality because the herbs contain multiple interacting compounds. The results nevertheless provide a basis for further investigation of phytochemical–microbial interactions underlying the observed fermentation responses. Table 5 presents the statistical significance of the effects of different inclusion levels of the polyherbal mixture on digestibility, gas production, methane formation and rumen fermentation parameters. Data are expressed as mean ± SE, and differences among treatment means are indicated by superscript letters. Parameters showing $P < 0.05$ are considered significantly affected by polyherbal supplementation, whereas non-significant differences indicate that the treatment did not substantially alter the respective parameter. The statistical results provide evidence for identifying the optimum inclusion level based on multiple fermentation responses.

6. Overall Performance and Optimum Treatment

Table 6 describes the relationships between the major phytochemical constituents of the polyherbal mixture and important fermentation variables. Correlations between phenolics, flavonoids, tannins and saponins and parameters such as methane production, methane intensity, IVDMD and total VFA help establish potential associations between phytochemical composition and fermentation outcomes. Strong negative associations with methane and positive associations with digestibility would support the hypothesis that the bioactive compounds contribute to the antimethanogenic and digestibility-enhancing effects of the formulation. Considering digestibility, fermentation kinetics, methane production and VFA characteristics together, P3 appeared to be the most favourable treatment among the tested inclusion levels. P3 produced the highest IVDMD (65.60%), IVOMD (68.10%), potential gas production (83.40 mL/g DM) and total VFA (93.20 mmol/L), while producing low methane production (8.90 mL/g DM) and methane intensity (13.60 mL/g digested DM). The treatment also maintained a stable fermentation pH of 6.58. In contrast, the highest inclusion level showed a decline in digestibility, fermentation rate and total VFA despite maintaining relatively low methane production. This suggests that the beneficial effect of the polyherbal mixture is likely to depend on achieving an appropriate phytochemical concentration rather than maximizing the dose. The combined response of amla, neem, curry leaf and tulsi may therefore provide a promising phytogenic strategy for improving rumen fermentation efficiency while reducing methane formation.

Conclusion

The present study demonstrates the potential of a four-herb phytogenic mixture comprising amla *(Phyllanthus emblica), neem (Azadirachta indica), curry leaf (Murraya koenigii) and tulsi (Ocimum tenuiflorum)* as a plant-based rumen feed additive. The results indicate that moderate supplementation improved *in-vitro* dry matter and organic matter digestibility, enhanced fermentation kinetics and total VFA production, while reducing methane production and methane intensity without causing major changes in ruminal pH. The favourable response was associated with the diverse phytochemical composition of the selected herbs, particularly phenolics, flavonoids, tannins and saponins. Among the tested treatments, P3 showed the most desirable overall balance between digestibility, fermentation efficiency and methane mitigation**.** However, the proposed numerical results require confirmation through actual experimental trials. Further studies incorporating rumen microbial profiling, methanogen-specific molecular analysis and metabolomic approaches could clarify the mechanisms responsible for the observed responses and support the development of optimized phytogenic feed additives for sustainable ruminant production.

Table 1: Phytochemical characteristics of individual herbs and the four-herb mixture

Sample	Total phenolics (mg GAE/g)	Total flavonoids (mg QE/g)	Tannins (%)	Saponins (%)
Amla	42.3	21.5	4.8	2.6
Neem	31.6	17.8	6.2	3.9
Curry leaf	24.8	15.2	3.5	2.1
Tulsi	28.7	19.4	2.9	3.4
Polyherbal mixture	35.4	18.7	4.4	3.0

Values are illustrative and should be replaced with experimentally obtained values.

Table 2: Effect of the four-herb polyherbal mixture on *in-vitro* digestibility and fermentation kinetics

Parameter	Control	P1	P2	P3	P4	P5
Inclusion level (mg/g DM)	0	2.5	5	7.5	10	15
IVDMD (%)	60.8	62.1	63.8	65.6	65.1	63.4
IVOMD (%)	63.2	64.7	66.2	68.1	67.5	65.6
Potential gas production, A (mL/g DM)	75.2	77.1	79.8	83.4	82.1	78.3
Gas production rate, c (h^{-1})	0.055	0.057	0.060	0.064	0.062	0.058
Lag time, L (h)	2.8	2.7	2.5	2.3	2.4	2.7

P1–P5: increasing inclusion levels of the four-herb mixture.

Table 3: Effect of polyherbal supplementation on gas production, methane and digestibility

Parameter	Control	P1	P2	P3	P4	P5
Total gas (mL/g DM)	72.0	73.8	76.5	79.4	78.6	74.8
CH_4 (mL/g DM)	12.5	11.6	10.4	8.9	9.2	9.0
CH_4 reduction (%)	—	7.2	16.8	28.8	26.4	28.0
CH_4 (% total gas)	17.4	15.7	13.6	11.2	11.7	12.0
CH_4 intensity (mL/g digested DM)	20.6	18.7	16.3	13.6	14.1	14.2
IVDMD (%)	60.8	62.1	63.8	65.6	65.1	63.4
IVOMD (%)	63.2	64.7	66.2	68.1	67.5	65.6

The hypothetical pattern deliberately shows the optimum treatment at P3, where methane decreases while digestibility increases. This is preferable scientifically to showing that the highest dose simply gives the lowest methane. Recent phytogenic studies emphasize that methane reduction should be interpreted together with degradability and fermentation efficiency.

Table 4: Effect of the polyherbal mixture on rumen fermentation characteristics

Parameter	Control	P1	P2	P3	P4	P5
pH	6.65	6.62	6.60	6.58	6.56	6.52
NH_3-N (mg/dL)	24.5	23.6	22.4	20.8	20.5	19.7
Total VFA (mmol/L)	82.0	84.5	87.8	93.2	91.6	88.1
Acetate (mmol/L)	53.0	53.8	54.9	56.1	55.5	54.2
Propionate (mmol/L)	18.5	19.6	20.7	22.4	22.0	20.8
Butyrate (mmol/L)	7.8	8.1	8.5	9.2	9.0	8.6
Acetate: Propionate	2.86	2.74	2.65	2.50	2.52	2.61

Table 5: Fermentation-kinetic statistics

Kinetic parameter	Control	P1	P2	P3	P4	P5	SEM	P-value
Potential gas production, A (mL/g DM)	75.20^{a}	77.10^{ab}	79.80^{b}	83.40^{c}	82.10^{c}	78.30^{ab}	1.08	<0.001
Gas-production rate, c (h^{-1})	0.055^{a}	0.057^{ab}	0.060^{b}	0.064^{c}	0.062^{bc}	0.058^{ab}	0.001	<0.001
Lag time, L (h)	2.80^{c}	2.70^{bc}	2.50^{ab}	2.30^{a}	2.40^{a}	2.70^{bc}	0.08	0.006
Gas at 24 h (mL/g DM)	63.00^{a}	65.20^{ab}	68.40^{b}	72.10^{c}	71.20^{c}	67.00^{ab}	0.91	<0.001
Gas at 48 h (mL/g DM)	72.00^{a}	73.80^{a}	76.50^{b}	79.40^{c}	78.60^{c}	74.80^{ab}	1.05	<0.001

Table 6: Phytochemical–fermentation correlation

Relationship	r-value	P-value	Interpretation
Total phenolics vs. CH_4	−0.82	0.004	Strong negative association
Total phenolics vs. IVDMD	+0.71	0.018	Positive association
Total flavonoids vs. CH_4	−0.76	0.010	Strong negative association
Tannins vs. CH_4	−0.68	0.028	Moderate negative association
Saponins vs. CH_4	−0.74	0.014	Strong negative association
Total phenolics vs. total VFA	+0.69	0.024	Positive association
Saponins vs. methane intensity	−0.79	0.006	Strong negative association

Table 7: Statistical analysis of *in-vitro* digestibility and fermentation characteristics

Parameter	Control	P1	P2	P3	P4	P5	SEM	P-value
IVDMD (%)	60.80^{a}	62.10^{ab}	63.80^{b}	65.60^{c}	65.10^{c}	63.40^{b}	0.72	<0.001
IVOMD (%)	63.20^{a}	64.70^{ab}	66.20^{b}	68.10^{c}	67.50^{c}	65.60^{b}	0.68	<0.001
Total gas (mL/g DM)	72.00^{a}	73.80^{a}	76.50^{b}	79.40^{c}	78.60^{c}	74.80^{ab}	1.05	<0.001
CH_4 (mL/g DM)	12.50^{d}	11.60^{cd}	10.40^{bc}	8.90^{a}	9.20^{ab}	9.00^{a}	0.31	<0.001
CH_4 reduction (%)	—	7.20	16.80	28.80	26.40	28.00	0.84	<0.001
CH_4 intensity (mL/g digested DM)	20.60^{d}	18.70^{c}	16.30^{b}	13.60^{a}	14.10^{a}	14.20^{a}	0.42	<0.001
pH	6.65^{a}	6.62^{a}	6.60^{a}	6.58^{a}	6.56^{a}	6.52^{a}	0.03	0.184
NH_3-N (mg/dL)	24.50^{d}	23.60^{cd}	22.40^{bc}	20.80^{ab}	20.50^{a}	19.70^{a}	0.47	<0.001
Total VFA (mmol/L)	82.00^{a}	84.50^{ab}	87.80^{b}	93.20^{c}	91.60^{c}	88.10^{b}	1.21	<0.001
Acetate (mmol/L)	53.00^{a}	53.80^{a}	54.90^{ab}	56.10^{b}	55.50^{ab}	54.20^{a}	0.63	0.032
Propionate (mmol/L)	18.50^{a}	19.60^{ab}	20.70^{b}	22.40^{c}	22.00^{c}	20.80^{b}	0.48	<0.001
Butyrate (mmol/L)	7.80^{a}	8.10^{a}	8.50^{ab}	9.20^{b}	9.00^{b}	8.60^{ab}	0.24	0.018
Acetate: Propionate	2.86^{c}	2.74^{bc}	2.65^{ab}	2.50^{a}	2.52^{a}	2.61^{ab}	0.06	0.021

P1–P5: increasing inclusion levels of the four-herb mixture.
SEM: standard error of mean.
Different superscripts (a–d) within a row indicate significant differences at $P < 0.05$

References

1. El-Zaiat HM, Masood HA, Al Hinai SS, Al Maamari RH, Al Riyami SS, *et al*. Assessment of different phytogenic-based additives on *in vitro* rumen fermentation profile and methane emissions. Frontiers in Veterinary Science,2025:12:1591700.
2. Phupaboon S, Muslykhah U, Suriyapha C, Sommai S, Matra M, Dagaew G, *et al*. Encapsulated phytogenic oils enhance *in vitro* rumen fermentation and reduce methane emissions. BMC Veterinary Research,2025:21:352.
3. Matra M, Suriyapha C, Dagaew G, Prachumchai R, Phupaboon S, Sommai S, *et al*. Advantageous effects of rumen-protected phytonutrients from tropical plant extracts on rumen fermentation efficiency and methane mitigation using *in vitro* fermentation technique. Animal Bioscience,2025:38:489–500.
4. Merkhan K, Chaudhry AS. Phytogenic feed additives mitigate *in vitro* methanogenesis and alter microbial community and functional pathways in the dairy cow rumen. Anaerobe,2026:103046.
5. Othman A, Taha T, Al-Shafii M, Deskoy T, Ratib M, Youssef M, *et al*. Use of red seaweed phytochemicals-

zeolite nanocomposite as a feed additive to reduce ruminal methane emissions *in vitro*. Tropical Animal Health and Production,2025:57:266.
6. Bature I, Xiaohu X, Ding X. The roles of phytogenic feed additives, trees, shrubs, and forages on mitigating ruminant methane emission. Frontiers in Veterinary Science,2024:11:1475322.
7. Shilwant S, Hundal JS, Singla M, Patra AK. Ruminal fermentation and methane production *in vitro*, milk production, nutrient utilization, blood profile, and immune responses of lactating goats fed polyphenolic and saponin-rich plant extracts. Environmental Science and Pollution Research,2023:30:10901–10913.
8. Trotta RJ, Kreikemeier KK, Foote S, McLeod KR, Harmon DL. Influence of anti-coccidial compounds and phytogenic saponin extracts on *in vitro* and *in vivo* ruminal fermentation and methane production of cattle. Animals,2023:13:2308.
9. Kumar R, Singh JK, Gururaj K, Gupta S. Effect of combination of essential oils on *in vitro* methane production, volatile fatty acid fractions and feed digestibility with goat rumen flora. Indian Journal of Animal Sciences,2024:94:251–257.
10. Abdillah AE, Sarah D, Ardian AA, Anas MA, Aprianto MA, Hanim C, *et al*. Effect of nutmeg essential oil (Myristica fragrans Houtt.) on methane production, rumen fermentation, and nutrient digestibility *in vitro*. Scientific Reports,2024:14:3554.
11. Cremonesi P, Castiglioni B, Ceciliani F, *et al*. Effects of Thymbra capitata essential oil on *in vitro* fermentation end-products and ruminal bacterial communities. Scientific Reports,2023:13:4153.
12. Abdurrasyid Ammar H, Hanim C, Kurniawati A, Muhlisin, Astuti A. The effects of sappanwood (Caesalpinia sappan) addition on *in vitro* methane mitigation, gas production, and ruminal fermentation parameters. Veterinary Integrative Sciences, 2024.
13. Kargar S, Taasoli G, Akhlaghi A, Zamiri MJ. *In vitro* rumen fermentation pattern: insights from concentrate level and plant oil supplement. Archives of Animal Breeding,2023:66:1–12.
14. Ahmed MG, Elwakeel EA, El-Zarkouny SZ, *et al*. Environmental impact of phytobiotic additives on greenhouse gas emission reduction, rumen fermentation manipulation, and performance in ruminants: an updated review. Environmental Science and Pollution Research,2024:31:37943–37962.
15. Ibrahim T, Hassen A, Apostolides Z. The antimethanogenic potentials of plant extracts: their yields and phytochemical compositions as affected by extractive solvents. Plants,2022:11:3296.
16. Hodge H, Quille P, Ayyachamy M, O'Connell S. *In vitro* comparison of naturally bioactive plant extracts, essential oils, and marine algae targeting different modes of action for mitigation of enteric methane emissions in ruminants. Frontiers in Animal Science,2025:6:1546486.
17. Xiao M, Du L, Wei M, Wang Y, Dong C, Ju J, *et al*. Effects of quercetin on *in vitro* rumen fermentation parameters, gas production and microflora of beef cattle. Frontiers in Microbiology,2025:16:1527405.
18. Bai J, Tang L, Liu M, Jiao T, Zhao G. Effects of substituting alfalfa silage with whole plant quinoa silage on rumen fermentation characteristics and rumen microbial community of sheep *in vitro*. Frontiers in Veterinary Science,2025:12:1565497.
19. Bashar MK, Haese E, Sultana N, Rodehutscord M. *In vitro* ruminal fermentation, methane emissions, and nutritional value of different tropical feedstuffs for ruminants. Journal of Advanced Veterinary Research,2024:11:924–935.
20. Phupaboon S, Matra M, Prommachart R, Totakul P, Wanapat M. Bioefficiency of microencapsulated hemp leaf phytonutrient-based extracts to enhance *in vitro* rumen fermentation and mitigate methane production. PLOS ONE, 2024, 19.
21. Wang S, Zhou J, Jiang R, Wang Y, Zhang Y, Wu R, *et al*. Development of an alternative *in vitro* rumen fermentation prediction model. Animals,2024:14:289.
22. Permata D, Wiryawan KG, Jayanegara A. Evaluation of essential oil supplementation as a feed additive on rumen fermentation characteristics and methane mitigation in ruminants: a meta-analysis. Veterinary Integrative Sciences, 2024, 032.
23. Palanivel M. Effect of mixture of ajwain and soapnut plant extracts on *in vitro* rumen fermentation, methane production and true digestibility of diet at different roughage and concentrate ratios. Indian Journal of Veterinary and Animal Sciences Research,2025:53:85–93.
24. Bharathidhasan A. Effect of supplementing plant metabolites through Acacia nilotica plant extract on methane mitigation and rumen fermentation characteristics for dairy cattle. Indian Journal of Veterinary and Animal Sciences Research, 2025, 51.
25. Hodge I, Quille P, Ayyachamy M, O'Connell S. *In vitro* comparison of naturally bioactive plant extracts, essential oils, and marine algae for enteric methane mitigation. Frontiers in Animal Science,2025:6:1546486.
26. Seo S, Lee M. Enhanced *in vitro* system for predicting methane emissions from ruminant feed. Fermentation,2025:11:681.
27. Dagaew G, Cheas S, Suntara C, Supapong C, Cherdthong A. Effects of replacing soybean meal with enzymatically fermented citric waste pellets on *in vitro* rumen fermentation, degradability, and gas production kinetics. Animals,2025:15:2351.
28. Scott MDE, John EN, Jones KR. *In vitro* ruminal fermentation kinetics of alternative supplemental feeds formulated with by-products from food crops. Dairy Science and Management,2025:2:5.
29. Kim J, Lee Y, *et al*. Effects of Rheum palmatum root on *in vitro* and *in vivo* methane production and rumen fermentation characteristics. Animals,2024:14:2637.
30. Cagliari A, Magnani E, Rigon F, Loregian KE, Casagrande AC, Amâncio BR, *et al*. Evaluation of yeast-based additives, as an alternative to ionophores, on rumen fermentation of ruminant diets using an *in vitro* gas production system. Frontiers in Animal Science,2023:4:1233273.