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**Short Communication**

# Effects of fenugreek (*Trigonella foenum-graecum*) seeds and areca nut (*Areca catechu*) powder supplementation on rumen fermentation and enteric methane production *in vitro*

**P. M. Nair[1], S. Kamboj[1], A. T. F. Jasmin[2], S. Arulkumar[1] and G. Mondal[1*]**

[1]Animal Nutrition Division, National Dairy Research Institute, Karnal - 132 001, Haryana, India: [2]Animal Biochemistry Division, National Dairy Research Institute, Karnal - 132 001, Haryana, India

## Abstract

**This study evaluated the effects of tannin source and saponin source, and their combination, on methane mitigation in buffalo using *in vitro* techniques. Areca nut powder (A), fenugreek seed powder (F), and their 1:1 combination (F+A) were mixed at concentrations of 0%, 2%, and 4% with a substrate of 60% roughage and 40% concentrate. Results showed significant reductions in total gas and methane production across treatments. Total gas production decreased significantly ($P<0.05$) with all treatments; reductions were most pronounced in the F+A combination (from 175.00 to 157.50 mL/g DM). Methane ($CH_4$) emission as a percentage of total gas also reduced significantly, with the most significant decrease observed in the F+A treatment (23.85% to 20.90%). Areca nut and fenugreek did not significantly affect digestibility. Both additives reduced acetate levels and increased microbial biomass production, with areca nut showing the highest microbial biomass production. Ammonia nitrogen levels significantly decreased with higher additive inclusion, indicating improved nitrogen retention. Ruminal pH remained stable across all treatments, supporting optimal rumen function. These findings suggest that areca nut and fenugreek, particularly in combination, have the potential to be methane mitigation strategies in livestock systems.**

**Keywords:** Areca nut, Enteric methane, Fenugreek seeds, *In vitro* analysis, Rumen fermentation

## Highlights

- Fenugreek seed powder + areca nut powder reduced $CH_4$ from 23.85% to 20.90%, the highest reduction
- Plant-based additives mitigate $CH_4$ without affecting rumen pH or digestibility

Methane is a potent greenhouse gas with a high global warming potential, although its atmospheric lifespan is shorter compared to other greenhouse gases. In recent years, the contribution of methane emissions from agriculture has doubled relative to the pre-industrial era, with livestock, particularly ruminants, being a significant source. Therefore, the mitigation of methane emissions from livestock is critical in combating climate change. One of the most effective nutritional strategies for reducing ruminal methane emissions involves the use of plant-based feed additives containing secondary metabolites, such as condensed tannins and saponins, which have demonstrated anti-methanogenic and anti-protozoal properties (Beauchemin *et al*., 2022).

Fenugreek (*Trigonella foenum-graecum*) seeds and areca nut (*Areca catechu*) are notable for their saponin and tannin content and offer additional benefits beyond methane reduction. Fenugreek is a well-known galactagogue that enhances lactation performance and promotes milk production through hormonal stimulation (Ravi and Joseph, 2020). It also exhibits various medicinal properties, including antidiabetic, anticancer, and antioxidant effects (Wani and Kumar, 2018). On the other hand, Areca nut has pharmacological activities such as antiparasitic, antioxidant, antihypertensive, and digestive-promoting effects (Peng *et al*., 2015). This study aims to evaluate the impact of Areca nut powder (tannin source) and fenugreek seed powder (saponin source) and their combination in different doses (0%, 2%, and 4%) on methane mitigation in buffalo using *in vitro* techniques, focusing on their impact on rumen fermentation and methane production.

The study was conducted at the National Dairy Research Institute, Haryana, India, using herbal feed additives (areca nut and fenugreek seeds) sourced locally from Karnal. Buffaloes from the institute's Livestock Research Centre were used for rumen liquor collection. The rumen liquor was collected via a fistula

*Corresponding Author, E-mail: gmondal1075@gmail.com

in the early morning before feeding and watering, filtered through muslin cloth, and used as inoculum for *in vitro* gas production experiments. Dried and ground areca nut, fenugreek seeds, and their combination at a 1:1 ratio were mixed at concentrations of 0%, 2%, and 4% with a substrate containing 60% roughage (dried maize fodder) and 40% concentrate (Table 1). Each concentration was tested in triplicate, with 9 syringes incubated in a water bath at 39°C (Menke and Steingass, 1988) for the test samples.

**Table 1. Ingredients of concentrate mixture fed during the experimental period.**

| S. No. | Ingredient | Parts (%) |
|---|---|---|
| 1 | Maize grain | 25.00 |
| 2 | Barley grain | 10.00 |
| 3 | Wheat bran | 10.00 |
| 4 | De-oiled rice bran | 5.00 |
| 5 | Gram chuni | 10.85 |
| 6 | Mustard oil cake | 13.00 |
| 7 | Soybean meal | 12.00 |
| 8 | Groundnut cake | 6.00 |
| 9 | Cotton seed cake (decorticated) | 5.00 |
| 10 | Mineral mixture | 2.00 |
| 11 | Common salt | 1.00 |
| 12 | Toxin binder | 0.15 |
| | **Total** | **100.00** |

The solution media for the incubation included micro and macro mineral solutions, rumen buffer, resazurin, and distilled water. After 24 hours of incubation, total gas production was recorded, and 2 mL of headspace gas was collected for methane analysis using gas chromatography. The GC was equipped with a flame ionization detector (FID) and a Porapak-Q column (1.5 m × 3.2 mm × 2 mm), with injector, column, and detector temperatures set at 40°C, 50°C, and 100°C, respectively. Nitrogen was used as the carrier gas (30 mL/min through the injector and column; 300 mL/min through the detector). A standard gas mixture (50% $CH_4$ and $CO_2$ ) was calibrated. The digestibility of dry matter (DM) and organic matter (OM) were evaluated, and microbial biomass production (MBP) and partitioning factor (PF) were estimated. Ammonia nitrogen ($NH_3$-N) was assessed as per the Kjeldahl method (AOAC, 1990), while volatile fatty acids (VFA) were analyzed after centrifugation (Erwin *et al.*, 1961).

MBP (mg) = Truly Degestrible Organic Matter (mg) – (2.2 × GasVolume(mL)

PF = Truly Digestible Organic Matter (mg)/Gas Volume (mL)

Results were statistically processed using SPSS version 16.0 with a significance level of P<0.05, using the Tukey test, and presented as mean and Standard Error Mean (SEM).

The effects of Fenugreek seeds, Areca nut powder, and their combination on rumen fermentation parameters have been evaluated based on total gas production, methane emission, *in vitro* digestibility, volatile fatty acids (VFA) production, microbial biomass production, ammonia nitrogen, and pH (Table 2).

In all treatments, including control (0% inclusion), total gas production decreased significantly as the inclusion levels of fenugreek and Areca nut increased. For fenugreek, gas production dropped from 171.7 mL/g DM at 0% inclusion to 158.3 mL/g DM at 4% inclusion, with a statistically significant difference (P=0.007). Similarly, areca nut reduced total gas from 165 mL/g DM at 0% to 147.5 mL/g DM at 4% (P=0.003). The combination of fenugreek and areca nut showed a significant reduction in gas production from 175 mL/g DM at 0% to 157.5 mL/g DM at 4% (P=0.001). This consistent reduction suggests that both additives can lower rumen fermentation activity, reducing ruminant gas production (Wadhwa *et al.*, 2020; Niu *et al.*, 2021).

Fenugreek did not significantly affect in-vitro dry matter digestibility (IVDMD) across the inclusion levels (P=0.43), with values remaining relatively stable between 56.1% and 58.8%. Areca nut showed a non-significant improvement in IVDMD (P=0.44), increasing from 58.67% at 0% inclusion to 62.27% at 4%. Similarly, the combination of fenugreek and areca nut did not yield significant differences (P=0.32). The slight increase in digestibility with areca nut may be attributed to its influence on rumen fermentation, possibly improving the breakdown of fibrous components. In-vitro organic matter digestibility (IVOMD) values did not differ significantly among the inclusion levels for fenugreek (P=0.23) or areca nut (P=0.67) and the combination (P=0.08). This indicates that while the additives altered some fermentation dynamics, they did not majorly impact the overall digestibility of organic matter. Bhatta *et al.* (2009) mentioned that the negative effect of tannins on fermentation and digestion could be related to the formation of tannin–carbohydrate and tannin–protein complexes that are less degradable (Jayanegara and Palupi, 2010) or are toxic to rumen microbes, especially the methanogens.

Methane production as a percentage of total gas

**Table 2. Effect of supplementation of fenugreek seed powder, areca nut powder, and their combination on *in vitro* fermentation parameters using a 60:40 roughage-to-concentrate (R:C) ratio.**

| | Fenugreek seed powder (F) | | | | | Areca nut powder (A) | | | | | F+A (1:1 ratio) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0% | 2% | 4% | SEM | *P*-value | 0% | 2% | 4% | SEM | *P*-value | 0% | 2% | 4% | SEM | *P*-value |
| Total gas (mL/g DM) | 171.70$^{a}$ | 164.30$^{b}$ | 158.30$^{c}$ | 3.45 | 0.01 | 165.00$^{a}$ | 155.83$^{b}$ | 147.50$^{c}$ | 3.80 | 0.00 | 175.00$^{a}$ | 165.83$^{b}$ | 157.50$^{c}$ | 3.17 | 0.00 |
| IVTDMD (%) | 58.00 | 58.80 | 56.10 | 1.13 | 0.43 | 58.67 | 57.21 | 62.27 | 4.24 | 0.44 | 62.08 | 61.65 | 63.30 | 1.04 | 0.32 |
| IVTOMD (%) | 66.09 | 63.48 | 63.71 | 1.51 | 0.23 | 65.15 | 64.17 | 66.40 | 1.66 | 0.67 | 65.85 | 61.32 | 63.53 | 1.39 | 0.08 |
| $CH_4$ (%) | 24.52$^{b}$ | 22.53$^{ab}$ | 21.15$^{a}$ | 0.68 | 0.05 | 25.72$^{c}$ | 23.81$^{b}$ | 22.65$^{a}$ | 0.75 | 0.04 | 23.85$^{b}$ | 22.26$^{ab}$ | 20.90$^{a}$ | 0.63 | 0.03 |
| $CH_4$ (mL/100 mg) | 4.21$^{c}$ | 3.70$^{b}$ | 3.28$^{a}$ | 0.03 | 0.04 | 4.24$^{c}$ | 3.71$^{b}$ | 3.34$^{a}$ | 0.03 | 0.03 | 4.17$^{c}$ | 3.69$^{b}$ | 3.29$^{a}$ | 0.03 | 0.03 |
| Acetate (mM) | 50.63$^{b}$ | 50.93$^{b}$ | 46.67$^{a}$ | 1.47 | 0.03 | 53.46$^{b}$ | 52.48$^{b}$ | 48.86$^{a}$ | 1.62 | 0.04 | 51.68$^{b}$ | 51.20$^{b}$ | 47.37$^{a}$ | 1.35 | 0.04 |
| Propionate (mM) | 13.53 | 13.59 | 14.67 | 0.81 | 0.54 | 13.35 | 14.57 | 15.67 | 0.89 | 0.07 | 14.29 | 15.45 | 16.41 | 0.75 | 0.08 |
| Butyrate (mM) | 8.78 | 8.80 | 9.34 | 0.43 | 0.67 | 8.47 | 9.06 | 10.85 | 0.47 | 0.08 | 9.01$^{a}$ | 9.75$^{a}$ | 11.27$^{b}$ | 0.40 | 0.05 |
| AP ratio | 3.74$^{a}$ | 3.76$^{a}$ | 3.18$^{b}$ | 0.03 | 0.04 | 4.01$^{a}$ | 3.60$^{b}$ | 3.12$^{c}$ | 0.04 | 0.04 | 3.62$^{a}$ | 3.31$^{b}$ | 2.89$^{c}$ | 0.04 | 0.04 |
| PF | 3.51$^{a}$ | 3.52$^{a}$ | 3.77$^{b}$ | 0.03 | 0.04 | 3.57 | 3.75 | 4.10 | 0.03 | 0.12 | 3.39 | 3.36 | 3.65 | 0.03 | 0.16 |
| MBP (mg) | 44.83$^{a}$ | 43.28$^{a}$ | 48.59$^{b}$ | 1.20 | 0.03 | 45.26$^{a}$ | 48.26$^{a}$ | 55.84$^{b}$ | 1.32 | 0.02 | 41.52$^{ab}$ | 38.50$^{a}$ | 45.63$^{b}$ | 1.10 | 0.04 |
| Ammonia N (mg/dL) | 13.21$^{b}$ | 11.62$^{a}$ | 10.06$^{a}$ | 0.82 | 0.02 | 12.45$^{b}$ | 10.52$^{a}$ | 9.55$^{a}$ | 0.90 | 0.04 | 12.65$^{b}$ | 10.77$^{a}$ | 9.80$^{a}$ | 0.75 | 0.04 |
| pH | 6.86 | 6.74 | 6.68 | 0.54 | 0.43 | 6.81 | 6.55 | 6.61 | 0.59 | 0.72 | 6.77 | 6.58 | 6.61 | 0.50 | 0.54 |

*In vitro* dry matter digestibility (IVDMD), *in vitro* organic matter digestibility (IVOMD), partitioning factor (PF), microbial biomass production (MBP), and acetate to propionate ratio (A/P), SEM: Standard Error Mean.
Means with different superscripts a, b and c in the same row differ significantly ($P<0.05$)

significantly decreased as fenugreek and Areca nut levels increased. Fenugreek reduced methane from 24.52% at 0% inclusion to 21.15% at 4% (P=0.046). Areca nut exhibited a similar reduction, with methane emissions decreasing from 25.72% at 0% to 22.65% at 4% (P=0.04). The combination of fenugreek and Areca nut showed the most significant reduction in methane, from 23.84% at 0% to 20.90% at 4% (P=0.03). This substantial reduction in methane indicates that both additives, particularly in combination, can be used to lower enteric methane emissions, thereby contributing to the mitigation of greenhouse gases in livestock systems. Methane expressed as mL/100 mg of DM followed a similar pattern, with fenugreek, areca nut, and the combination all showing significant reductions in methane production (P=0.03 for all treatments). This further strengthens the case for using these feed additives in methane mitigation strategies. Studies have shown that when *Areca catechu* is included at a rate of 2% in total mixed rations (TMR), it effectively inhibits methane production due to its high concentration of bioactive compounds such as condensed tannins and saponins, which contribute to its methane-reducing properties (Wadhwa *et al.*, 2020). Similarly, fenugreek, which contains 3-5% saponins, has been found to enhance *in vitro* rumen fermentation variables while reducing methane emissions (Niu *et al.*, 2021). A study conducted in India with supplementation of herbal additives, including fenugreek, significantly reduced *in vitro* methane production and improved total volatile fatty acid concentration in rations with varied roughage-to-concentrate ratios (Singh *et al.*, 2024). The study by Rejil *et al.* (2008) investigated the effects of dietary supplementation with raw and roasted fenugreek seeds on methane emissions in cattle. The findings indicated that both forms of fenugreek supplementation influenced methane production, although the specific effects varied based on the form of fenugreek used.

Acetate levels decreased significantly

with increasing levels of fenugreek (P=0.03), dropping from 50.63 mM at 0% to 46.67 mM at 4%. Areca nut also showed a similar reduction (P=0.04), decreasing values from 53.46 mM at 0% to 48.86 mM at 4%. The combination of fenugreek and Areca nut further reduced acetate levels significantly (P=0.04), indicating a shift in fermentation patterns. Propionate production did not vary significantly across the treatments for fenugreek (P=0.54). Areca nut and the combination treatment showed trends toward increased propionate production at higher inclusion levels. However, the results were not statistically significant (P=0.07 for the areca nut and P=0.08 for the combination). This indicates a shift towards more propionate production, which benefits animal energy efficiency. Butyrate production showed a slight, non-significant increase across treatments with fenugreek (P=0.67) and areca nut (P=0.08). The combination treatment showed near-significance (P=0.052) at 4% inclusion, suggesting that these additives might promote butyrate production, which plays a role in rumen health and epithelial cell growth. Managing hydrogen production in the rumen is crucial when designing strategies to mitigate methane emissions in ruminants. Reducing $CH_4$ production can be achieved by limiting the reactions that release $H_2$ or by promoting alternative pathways that utilize $H_2$, thereby minimizing its availability during rumen fermentation (Sinz *et al.*, 2018). The A: P ratio decreased significantly across all treatments (P=0.04 for all), reflecting the lower acetate and stable or increasing propionate levels. This shift towards propionate is generally considered beneficial for animal production, leading to less hydrogen available for methane formation.

Microbial biomass production (MBP) was highest in the Areca nut treatment, ranging from 45.26 mg at 0% to 55.84 mg at 4%. Fenugreek led to an increase in MBP from 44.83 mg to 48.59 mg. The combination had lower microbial biomass than Areca nut alone, with values between 41.52 mg and 45.63 mg. This suggests that the Areca nut better supports microbial growth in the rumen. Ammonia nitrogen concentrations significantly decreased with increasing fenugreek inclusion, from 13.21 mg/dL at 0% to 10.06 mg/dL at 4% (P=0.02). A similar trend was observed with areca nut (P=0.04), where $NH_3$-N levels dropped from 12.45 mg/dL at 0% to 9.55 mg/dL at 4%. The combination treatment also resulted in a significant decrease in ammonia nitrogen (P=0.042). The reduction in ammonia nitrogen suggests improved nitrogen retention in the rumen, potentially reducing nitrogen losses and enhancing feed efficiency. Saponins in fenugreek seeds possess antimicrobial properties, inhibiting the growth of ciliate protozoa in the rumen. This suppression increases the bacterial population and enhances fermentation activity (Patra and Saxena, 2009).

No significant differences in ruminal pH were observed across any of the treatments. Fenugreek (P=0.43), areca nut (P=0.72), and the combination (P=0.54) all maintained stable pH values throughout the experiment, which is essential for maintaining optimal rumen function and avoiding acidosis.

Fenugreek seeds and Areca nut powder showed beneficial effects on rumen fermentation, with a consistent reduction in methane emissions and improved digestibility. However, combining the two provided the best balance across all parameters, particularly in methane reduction, VFA production, and maintaining digestibility. The combination also exhibited more consistent performance across concentrations, showing its potential for improving rumen fermentation efficiency while reducing environmental impact through lower methane emissions. However, *in vivo*, studies evaluating these combinations under practical feeding conditions are necessary to draw definitive conclusions.

**Conflict of interest:** The Authors have no competing interests to declare.

**Author's contribution:** PMN: Performed all experiments and data analysis and wrote the manuscript; SK, ATFJ: Helped in editing the manuscript; SAK: Helped in data analysis; GM: Helped in experimental concept, design, and final editing of the manuscript.

**Data availability statement:** All data supporting the findings of this research are included in this paper.

**Ethics approval for animal trial:** Approval was obtained from the Institute Animal Ethics Committee with IAEC approval no. 50-IAEC-23-05.

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