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Effect of *Azadirachta indica* leaf extract on *in vitro* enteric gas production, protozoan, bacteria and methanogen populations in cattle rumen

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Abstract

Several mitigation approaches including drugs, antibiotics, vaccines and chemical supplements have been adopted to mitigate methane emission from ruminants but found to be toxic to the host animal and sometimes expensive. This work studied the effect of methanolic extract of neem (*Azadirachta indica*) leaf as a cost effective and nontoxic supplement in cattle feed. The study determined the effect of increasing doses of neem leaf extract on bacterial, protozoa, methanogen population and total gas production in 24hrs of *in vitro* incubation. Treatments were designed to 9 doses of percentage methanolic extract of neem leaf (0, 10, 20, 30, 40, 50, 60, 70, and 80 %) supplemented to the cattle feed. Increased percentage of neem leaf extract resulted in significant ($p < 0.05$) decreased in total gas production. The highest inhibitory effect was recorded at 60% neem extract which reduces total gas produced from 23. 47 mL/g DM to 4.23 mL/g DM. The least inhibitory effect at 10% neem extract reduces gas production to 20.43 mL/g DM. Increased percentages doses of neem leaf extract also resulted in significant decrease ($p < 0.05$) in protozoa and methanogen counts as compared with the control. However, neem leaf extract at 10 – 20 % increased total bacteria count to 26. 67 × 10^6 CFU/mL as compared to control (25.33 × 10^6 CFU/mL) but increased percentage of neem leaf at 30% and above reduced total bacteria count as compared to the control. Therefore, this study shows that methanolic extract of neem leaf is a promising feed additive as rumen modifying agent. It has the potential to reduce enteric gas production and consequent emission from ruminants.

Keywords: *Azadirachta indica*, methanogens, gas production, rumen, cattle
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Introduction

A multitude of gases cover the earth, warming it and allowing life to exist there. The earth wouldn't support life and would be 20 to 30°C cooler without the gaseous layer that keeps it heated. However, there is a worldwide climatic shift occurring due to global warming. This is because the concentration of gases increases as they cover the world, increasing global temperatures. A portion of the solar energy that reaches the planet's surface is reflected back into space as invisible infrared radiation. The world warms as a result of the absorption of this infrared radiation by gases such as carbon dioxide, methane, and nitrous oxide (Gupta *et al*., 2014). These gases, often known as greenhouse gases, are the primary causes of global warming.

Agricultural sector is responsible for 3-4% of global gross domestic product (GDP). With growing world population, demand for meat and dairy product is bound to increase. Such increase in demand comes at the expense of growing carbon footprints. This is because meat and dairy product rely on ruminant animals which produce 80, 000,000 tons of methane annually. These ruminants are responsible for 30% global anthropogenic methane emissions (Arndt *et al*., 2021).The rumen hosts diverse group of microorganisms including bacteria, fungi, protozoa and methanogens. These organisms degrade plant material into volatile fatty acids, ammonia, hydrogen and carbon dioxide. Methanogens in the rumen then use hydrogen to reduce carbon dioxide to methane. Methane production constitute about 2-12% of total energy loss to the ruminants. This energy can be redirected towards meat or milk production and ensure better environmental sustainability as well (Patra and Yu, 2015).

Ruminant methane emissions have been reduced by the use of a number of mitigating strategies. These consist of medications like antibiotics, vitamins, feed additives, and vaccines. Vaccine administration, however, is costly and complicated. Antibiotic residues in animal products like milk and meat constitute a risk to human health, the use of antibiotics as feed additives in ruminants has been outlawed in the European Union (Papatsiros *et al*., 2014). Use of chemical feed additives to lower rumen methane production can either be hazardous to the host animal or only temporarily affect methane output. Chemical residue of these feed additives poses a concern to human health because it is present in meals derived from animals. These challenges prompted the research on feed additive technology that uses natural plant products (Honan *et al*., 2021).

Plant secondary metabolites (PSM) are employed as defense mechanisms against insect and microbial invasion. These PMS include alkaloids, phenols, tannins, flavonoids and saponins can influence the rumen microbial community and enhance nitrogen metabolism. Additionally, they are employed as antimicrobials to lower the populations of protozoa and methanogens thereby reducing methane production (Wanapat *et al*., 2009). Research is ongoing on the potential use of plants and their bioactive component as modifiers of rumen microbial fermentation to inhibit enteric methane production. The present work determines the effect of methanolic extract of neem (*Azadirachta indica*) leaf as a nontoxic supplement in cattle feed to reduce enteric methane production.

Materials and Methods

Preparation of Sample

Fresh matured neem leaves were collected from neem tree and washed with water to remove dust and dirt. Leaves were removed from its stem and air dried at room temperature for 3days. The dried leaves were then grounded into powder using a grinder and sieved to pass through 1mm sieve following the methods demonstrated by Deeni *et al*., 2002.

Preparation of Plant Extract

Three days of maceration were used to extract 50g of the powdered dried leaves from a conical flask containing 500 mL of methanol solvent. Crude extract was obtained by evaporating the methanol solvent under reduced pressure using a Yamato Rotary Evaporator, model RE 801 (Anokuwuru *et al*., 2011).

Preparation of Inoculum

Following the techniques demonstrated by Mould *et al*., 2005, ruminal content was collected from freshly slaughtered cow from a slaughterhouse in Lapai. The collected fluid was pooled and transferred to a pre-warmed flask and immediately transported to the laboratory. This was then filtered through four layers of cheese cloth to remove feed particle. The filtered fluid was transfer into a pre-warmed flask in a water bath at 39^{o}C and maintained under continuous flushing with CO_2 until use (Bueno *et al*., 2005).

Treatments Preparation

Five grams of dry matter (DM) of the substrate were added to a 500 millilitre conical flask, and different percentages of

plant extract (10%, 20%, 30%, 40%, 50%, 60%, 70%, and 80%) were also added. Each conical flask was filled with 300 mL of phosphate buffer solution and 150 mL of ruminal fluid (pH adjusted to 6.8) and flushed with CO_2 to maintain anaerobiosis. After corking each flask, a syringe was attached to the top for collecting gas. As a result, the experimental design consists of eight flasks, each holding three copies of the substrate, neem leaf extract (in varying concentrations), and ruminal fluid. In three repetitions, the blanks contained ruminal fluid and buffer, while the control group also had three duplicates of ruminal fluid, substrate, and buffer solution. All of these were incubated for 24 hours at 39 °C using the methods adopted by Al-Marzooqi *et al.* (2021).

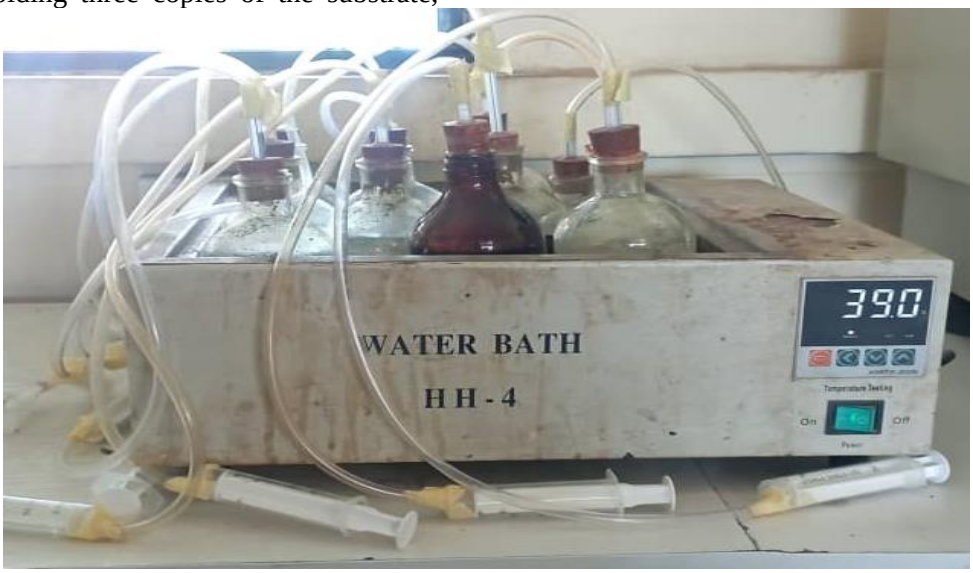

Figure 1: Gas Collection and Measurement

Every conical flask was equipped with a syringe for collecting gas, so any gas generated during fermentation would flow straight into the syringes. The displacement of the piston in each syringe was used to determine the total gas volume in each syringe (Figure 1).

Estimation of Methanogen Count (CFU/mL)

After 24hrs incubation, each of the individual test flasks was serially diluted up to the 6^{th} dilution. 1mL from each of the 6^{th} dilution test tube was transferred onto compounded mineral enriched medium which was prepared following the methods of Gosh *et al* (2014) and Manimegelai *et al* (2014). The sample and the plate were mixed by moving the plate in a circular motion. Each of the plates were prepared in 3 replicates and incubated anaerobically at 37^{o}C for 7 days.

Estimation of Protozoa Count (Cells/mL)

Following a 24-hour incubation period, protozoa were counted in each treatment. Two millilitres of each of the six dilutions of the flask's contents were transferred into a screw-capped test tube that held 5 mL of the formalin-normal saline solution (20 mL of formalin in 100 mL of normal saline). Each test tube also contained two drops of brilliant green dye (2g of brilliant green and 2 mL of glacial acetic diluted in 100 mL of distilled water). This was well combined and let to rest at room temperature for the entire night.

Two drops of the liquid were placed onto a slide and covered with cover slip in order to count the protozoa. For every test tube, three duplicates of this were manufactured. Every slide was examined at a magnification of ×100. For each slide, a total of 25 microscopic areas were examined to determine the number of protozoa cells (Kargar *et al.*, 2023).

Estimation of Bacterial Count (CFU/mL)

Each test flask was serially diluted up to the sixth dilution following a 24-hour incubation period and gas collection. A nutrient agar

petri dish was filled with 1 mL from each of the test tubes used in the sixth dilution. The agar plate was rotated in a circular manner to fully combine the sample. Moreover, this was done three times. This was further kept in an incubator anaerobically for 24 hours at 37 °C (Kumar *et al.,* 2019). At the end of incubation, visible colonies were counted as the number of bacteria CFU/mL.

Statistical Analysis

To examine the impact of increasing concentrations of neem leaf extract on the rumen microbial population and overall gas production, all data were statistically analysed using one-way ANOVA with Dunchan multiple range, posthoc IBM SPSS statistics (version 26), and correlation analysis to examine the relationship between decreased populations of protozoa and methanogens and gas production.

Results and Discussion

Inhibition and Estimation of Enteric Gas Production

Figure 2 shows total gas produced after 24hrs in vitro fermentation supplemented with neem leaf extract. The figure shows a significant ($p < 0.05$) decrease in total gas production with increase in percentage neem leaf extract. The highest inhibitory effect was observed at 60% reducing gas production from 23.47mL/g DM to 4.23mL/g DM while the lowest inhibitory effect was observed at 10% reducing total gas production to 20.43mL/g DM. No gas was produced at 70% and 80% of added neem leaf extract.

Estimation of Methanogen Population

Figure 3 shows total methanogen count after 7days incubation. In this study, total methanogen count decreases significantly ($p < 0.05$) with increase in percentage neem leaf extract. The highest inhibitory effect is seen at 50% reducing total methanogen count from 10.33×10^6 CFU/mL in the control to 0.67×10^6 CFU/mL while the lowest inhibitory effect is seen at 10% neem leaf extract reducing total methanogen count to 9.0×10^6 CFU/mL. No growth was observed at 60%, 70% and 80% of neem extract added.

Estimation of Protozoa Population

Figure 4 shows the total count of protozoa after 24hrs fermentation. Decreased total protozoa count was observed in the treatments from the lowest percentage 10% to the highest 80%. Supplementing with extract of *A. indica* leaf at 10% reduced total protozoa count from 14.0×10^6 cells/mL to 10.0×10^6 cells/mL which is the lowest inhibitory effect while at 80% total protozoa count was reduced from 14.0×10^6 cells/mL to 1.3×10^6 cells/mL which is the highest inhibitory effect.

Estimation of Total Bacterial Population

Figure 5 shows total bacterial count when extract of neem leaf at differing percentages were supplemented to cow feed in an in vitro experiment. Figure 4 shows an increase in total bacterial count from 25.33×10^6 CFU/mL to 27.33×10^6 CFU/mL when 10% *A. indica* extract was added. Addition of 20% extract also increased total bacterial count from 25.33×10^6 CFU/mL but decreased total bacterial count from 27.33×10^6 CFU/mL to 26.67×10^6 CFU/mL.

Effect of Neem Leaf Extract on rumen Microbial Population and Gas Production

Table 1 shows the effect of supplementing cow feed with neem leaf extract on bacteria, protozoa, methanogens and gas production in the rumen at varying percentages. Supplementation at percentages bearing superscript of the same alphabets has no significant difference ($p > 0.05$).

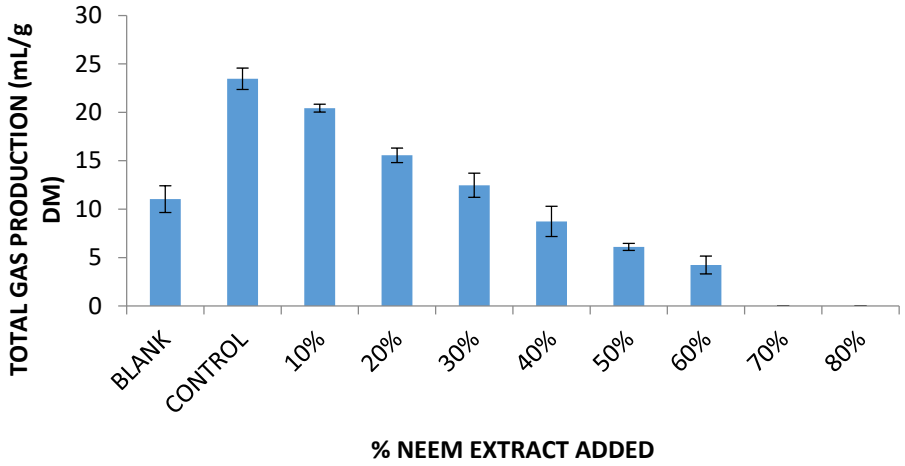

Figure 2: Total gas production after 24hrs fermentation. The error bars represent the standard deviations of the triplicate data.

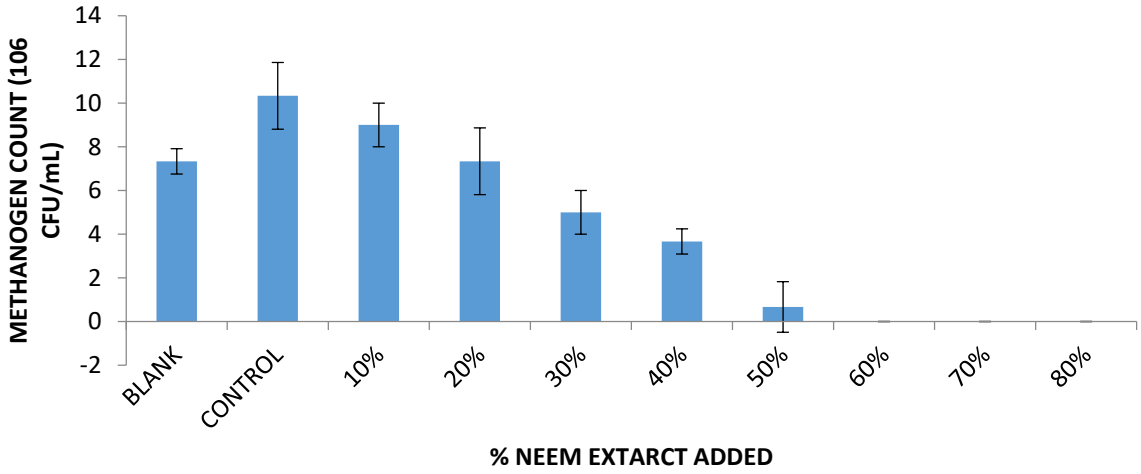

Figure 3: Total Methanogen Count after 7 days Incubation. The error bars represent the standard deviations of the triplicate data.

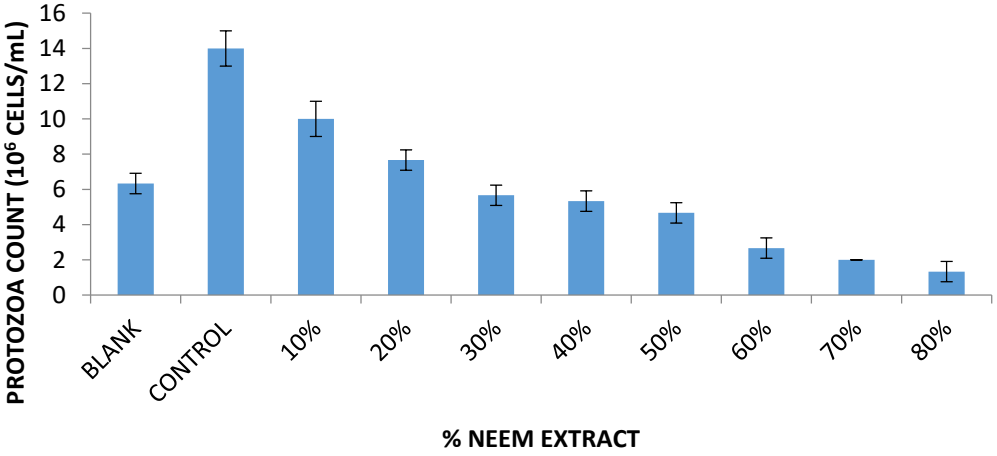

Figure 4: Total Protozoa count after 24hrs incubation. The error bars represent the standard deviations of the triplicate data.

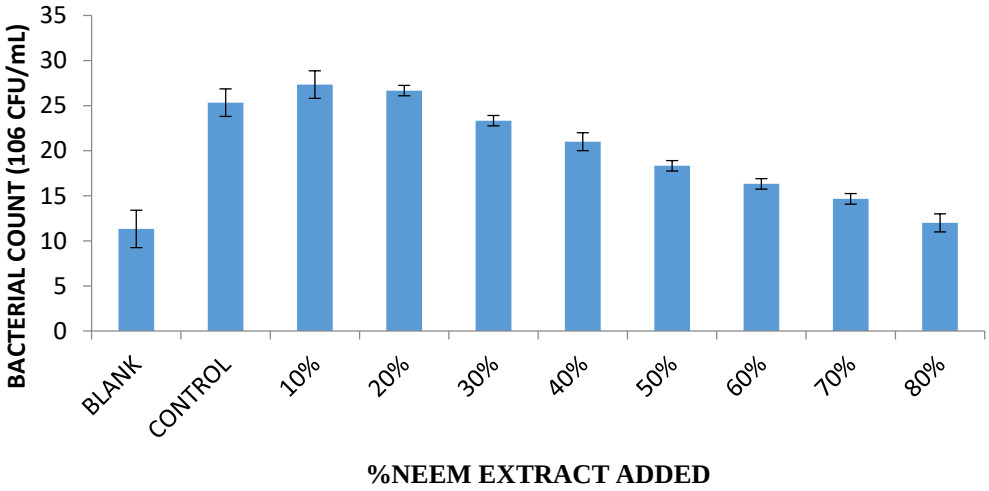

Figure 5: Total Bacterial count after 24hrs incubation. The error bars represent the standard deviations of the triplicate data.

Relationship between Reduced Protozoa, Methanogens and Gas Production in the Rumen

Table 2 shows the relationship between protozoa, methanogen and gas production. There is very strong positive correlation (r = 0.91) between protozoa and methanogens and also a very strong positive correlation (r = 0.94) exists between methanogen and gas production.

Table 1: Effect of Neem Leaf Extract on rumen Microbial Population and Gas Production

%Neem Extract	Bacteria(CFU/mL)	Protozoa(cells/mL)	Methanogen (CFU/mL)	Gas Production (mL/g DM)
0%	18.33 ± 7.84^{bc}	10.17 ± 4.26^{d}	8.83 ± 1.90^{c}	17.25 ± 6.90^{de}
10%	27.33 ± 1.52^{d}	10.00 ± 1.00^{d}	9.00 ± 1.00^{d}	20.43 ± 0.41^{e}
20%	26.67 ± 0.58^{d}	7.67 ± 0.58^{cd}	7.33 ± 1.52^{c}	15.56 ± 0.75^{d}
30%	23.33 ± 0.58^{cd}	5.67 ± 0.58^{c}	5.00 ± 1.00^{bc}	12.47 ± 1.25^{cd}
40%	21.00 ± 1.00^{c}	5.33 ± 0.58^{bc}	3.67 ± 0.58^{b}	8.73 ± 1.56^{c}
50%	18.33 ± 0.58^{bc}	4.33 ± 0.58^{b}	0.67 ± 1.57^{ab}	6.10 ± 0.36^{bc}
60%	16.33 ± 0.58^{b}	2.67 ± 0.58^{ab}	0.00 ± 0.00^{a}	4.23 ± 0.93^{b}
70%	14.67 ± 0.58^{ab}	2.33 ± 0.58^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
80%	12.00 ± 1.00^{a}	1.33 ± 0.58^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Values bearing the same alphabet in the same column are not significantly different ($p > 0.05$)

Table 2 Relationship between Reduced Protozoa, Methanogens and Gas Production in the Rumen.

Parameter	Protozoa	Methanogen	Gas Production
Protozoa	1		
Methanogen	0.91*	1	
Gas production	0.96*	0.94*	1

*Significant

Discussion

Figure 2 and 3 shows a significant decrease ($p < 0.05$) in methanogen population and total gas production from 10% to 60% neem extract added. Reduced methanogen and gas production has a positive effect on the environment because methane is one of the gases produced in the rumen and is a potent greenhouse gas and reducing its production in the rumen and consequent emission into the environment contributes to overall greenhouse gas emission. Methane production by methanogens is sometimes seen as an energy loss, this energy can be redirected towards meat or milk production potentially improving feed efficiency in the host animal.

On the other hand, reduced methanogen population and gas production in the rumen can have a number of negative impacts to the animal. Because methanogenesis is part of the natural process of fermentation to degrade complex material into simple compounds, reduced methanogen population can affect this process and in turn nutrient utilization from the feed. Methanogens constitute a part of the rumen microbiome, altering their population can affect overall microbial balance and normal processes in the rumen. These can negatively impact overall animal health performance.

Different studies have shown the effect of plant secondary metabolite on methanogens and methane production. Taethaisong *et al*., (2023) showed that supplementing 6% neem leaf + 15% polyethylene glycol in goats reduced methanogen population and net gas production. An in vitro study by Rira *et al.,* (2015) supplemented *Acacia cyanophylla* at 60% and 30% reduced methane production by 37.5 and 56.2% respectively due to high content of tannins that reduced methanogen population. An *in vitro* and *in vivo* study carried out by Holtshausen *et al*., (2009) on saponin derived from *Yucca schidigera* and *Quillaja saponaria* showed a 6-26% reduction in methanogenesis on *in vitro* studies but *in vivo* studies using whole plant powders at 10 g/kg DM did not show an impact on rumen fermentation.

Four different plants containing flavonoids were studied *in vitro* for methane inhibition using rumen fluid from cows; methane production was reduced by 39-48% in all treatments (Kim *et al*., 2015). Sheep diets supplemented with mulberry leaves containing flavonoids at ~1.3 g/kg DM did not inhibit methane production to a detectable level, but digestibility was increased**.** Four different plants containing flavonoids were studied *in vitro* for methane inhibition using rumen fluid from cows, methane production was reduced by 39-48% in all treatments (Kim *et al*., 2015). Sheep diets supplemented with mulberry leaves containing flavonoids at ~1.3 g/kg DM did not inhibit methane production to a detectable level, but digestibility was increased (Chen *et al.*, 2015).

In 2008, Kamra *et al.,* screened 93 plants extract for their potential to inhibit methanogenesis and ciliate protozoa in an *invitro* gas production test using buffalo rumen liquor as the inoculum. 20 extracts exhibited methanogenic activity reducing methanogens and reduced gas production up to 50%. Taethaisong *et al.,* (2022) reported a decrease in methanogen population when Goat feeds were supplemented with 6% purple foliage neem + 3% sunflower oil. Yusuf *et al*. (2017) reported that supplementing goat's feeds with leaves and whole plant of *Andrographis paniculata* had no significant effect on population of protozoa and methanogens.

In this study, total protozoa population was reduced significantly ($p < 0.05$) with increase in percentage of neem leaf extract (Figure 4). This inhibitory effect on protozoa count could be attributed to the presence of saponins and other plant secondary metabolite present in *A. indica*. Saponins interact with sterols found in protozoan membranes which lead to cell membrane destruction causing the leakage of cellular content. Flavonoids also affect protozoa by inhibiting cell wall synthesis or nucleic acid synthesis (Lila *et al.,* 2003). Due to the presence of tannins in neem leaf extract, tannins can enter into the cell membrane of

protozoa and cause cell membrane disruption in the organisms.

Reduced protozoa population is beneficial to the animal as it increases milk yield and milk protein to fat ratio in dairy cows (Hart *et al.*, 2007). Because protozoa actively feed and engulf bacteria make their presence undesirable in the rumen. Methanogens live in close association with ciliate protozoa attached to the outer surface of protozoa thereby providing habitat for methanogens (Bunglavan, 2014), thus, reduced protozoa population in the rumen help to indirectly inhibit methane production because the methanogens lose their partner and methane synthesis is partially inhibited. However, total elimination of protozoa is undesirable as protozoa are responsible for number of fibrolytic activities in the rumen. When protozoa populations are very low, it affects fibre digestion which impair breakdown of feed component and this can affect overall digestion. Protozoa are also involved in breakdown of complex polysaccharides into simple sugars which can be easily absorbed by the host animal, thus a decreased in population of protozoa can affect fermentation by-product and in turn alter nutrient utilization by the host animal.

Different studies have shown the effect of plant secondary metabolites on protozoa population in the rumen. Taethaisong *et al*. (2023) reported a reduction in protozoa and methanogens when goats were fed 6% neem leaf + 15% polyethelene glycol. In 2022, Taethaisong *et al*, reported decreased protozoa and methanogen population when goat feeds were supplemented with 6% purple foliage neem + 3% sunflower oil.

A 2019 study by Anuson *et al*., showed a reduced protozoa population and methane production in cattle diets supplemented with *Piper sermentosum* leaf powder. Hermandez *et al*., 2012 confirmed that flavonoids can be used to reduce protozoa counts when supplemented in animal diets. Paula *et al.*, 2010 also showed that protozoa in water buffaloes can be reduced by increasing the level of flavonoid compounds. Anantasook *et al*., (2014) showed that feeding diary steers *Samanea saman* (rain tree), a tropical leguminous plant in Mexico resulted in reduced protozoa population and consequently methane emission. The authors concluded that the presence of saponins in the plant helped decrease protozoa population and probably methanogens which resulted in upto 50% decreased methane synthesis. However, Cobellis *et al*. (2016) reported that supplementing sheep feeds with rosemary (*Rosmarinus offcinalis)* leaves and essential oil did not affect abundance of total protozoa or *Ruminococcus flavefaciens* a bacterium, but the leaves decreased the abundance of archaea.

Figure 5 shows that supplementation with neem leaf extract at 10% and 20% increased bacteria population initially but higher percentage of extracts had a significant ($p < 0.05$) inhibitory effect on total bacterial count with the highest inhibition seen at 80% extract reducing total bacterial count to 12.00×10^6 CFU/mL. The initial increase in bacterial population could be attributed to less susceptibility of bacteria to saponins. Saponins form complexes with sterols and bacterial membranes lack sterols. Figure 4 shows a reduced population of protozoa at 10% extracts added, and because protozoa actively feed and engulf bacteria, reduced protozoa mean reduced engulfment of bacteria by protozoa.

Having increased levels of bacterial population in the rumen is beneficial to the host animal because bacteria are also important in breaking down complex fiber in the rumen, thus, an increase in bacterial population in the rumen will result in increased fiber digestion and better utilization of feed. Increased rumen bacteria also lead to increased production of volatile fatty acids and other fermentation by-products since bacteria are major contributors of fermentation in the rumen. This consequently leads to enhanced nutrient absorption by the host animal which can also improve overall animal performance. Increased bacteria also help stabilize rumen pH. However, a reduced population of bacteria is not desirable because it can impair fiber digestion, decrease fermentation and

contribute lower nutrient utilization which can affect overall animal performance.

In a recent study by Taethaisong *et al*., (2023), adding 6% neem leaf + 15% polyethylene glycol in goat feeds increased bacteria population but reduced protozoa and methanogen population. 6% purple neem foliage + 3% sunflower oil as feed supplement increased total bacterial population in ruminants. Yang *et al*., (2009), reported 24% increase in bacterial population when neem oil was supplemented in cattle feed. Yusuf *et al*., (2017) reported that supplementing goats feeds with leaves and whole plant of *Andrographis paniculata* increased populations of bacteria but had no significant effect on population of protozoa and methanogens. A (2022) study by Jahani *et a*l., showed that diary calves receiving 4mL/day of IMPE herbal extract mixture (containing coneflower, yarrow, peppermint and citrus essential oil) had abundance of bacterial population in the rumen than calves with no additives. In an earlier study by Wanapat *et al*., (2013), supplementing cattle feeds with 100g/d lemongrass plus 10g/d peppermint with 40g/d garlic powder decreased population size of total viable and protoelytic bacteria and protozoa while decreasing methane production.

In this study, table 2 shows that there is very strong correlation (0.94) between reduced methanogen and reduced gas production. This is because methane gas is one of the by-products of fermentation in the rumen and methanogens are organisms that produce this gas, thus a reduction in the population of methanogens means a reduction in methane gas production and an increase in methanogen population means an increase in methane production. There is also strong correlation (0.91) between reduced protozoa and reduced methanogens. This may be due to the fact that some methanogens are found in symbiotic relationship with protozoa where the protozoa gives shelter to the methanogens, thus a reduction in protozoa population will affect methanogen population because they also lose their host.

Conclusion and Recommendation

Methane emission from ruminants can be of concern to the environment as methane is a potent greenhouse gas. Increased concentrations of this gas in the atmosphere contribute to global warming which is a global problem. Production of this gas in the rumen also constitutes energy loss in the animal which can be directed towards other more productive process such as meat or milk production. Therefore, inhibiting methane emission from ruminants can help fight the war against climate change. In this study, photochemical screening of *Azadirachta indica* (neem) leaf extract shows the presence of plant secondary metabolite in substantial quantity. These PSM have showed to possess antimicrobial activity in various studies. This study found that supplementation with *A. indica* at varying percentages, significantly ($p < 0.05$) reduced microbial population as compared to those of controls and consequently reduced the amount of total gas produced. It shows that supplementing at 10% - 20% reduced gas production and methanogens and protozoa population while increasing bacterial population which is beneficial to the animal because bacteria are required for protoelytic activity. However, higher percentages above 20% decreased bacterial population, which is undesirable in the rumen because reduced bacteria population affects proteolytic activity which consequently affects overall animal health and performance.

Recommendation

Future studies are recommended to evaluate other plants for plant secondary metabolites which can be used as feed additives to mitigate production of methane in ruminants. Further studies are also required to quantify the amount of methane produced from total gas production.

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