INTERNATIONAL JOURNAL OF RESEARCH AND SCIENTIFIC INNOVATION (IJRSI)
ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XIII Issue V May 2026

Effect of Corn Stover and Red Calliandra Ratios on Rumen pH, Microbial Populations, and Methane Production (In Vitro)

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DOI: https://doi.org/10.51244/IJRSI.2026.1305000067

Received: 02 May 2026; Accepted: 08 May 2026; Published: 29 May 2026

ABSTRACT

This study aims to determine the effect of various ratios of corn stover *(Zea mays)* and Red Calliandra *(Calliandra calothyrsus)* on pH, Bacterial Population, Protozoa, and Methane Gas in silage as an indicator of fermentation quality. This study was conducted using a Completely Randomized Design (CRD) with four treatments: P1 (90% corn stover + 10% Red Calliandra), P2 (80 corn stover + 20% Red Calliandra), P3 (70% corn stover + 30% Red Calliandra), and P4 (60% corn stover + 40% Red Calliandra). Data were analyzed using analysis of variance (ANOVA) followed by Duncan's multiple range test. The results showed that variations in the ratio of corn stover and red Calliandra did not significantly affect the bacterial population ($3.17–3.352 \times 10^9$ Cell/mL) and methane production (5.55–6.141 mM) ($P > 0.05$). In contrast, the protozoa population significantly affected by the treatment ($4.23–5.332 \times 10^5$ cells/mL) and rumen pH significantly affected by the treatment (6.836–6.888) ($P \leq 0.05$) with the addition of red Calliandra. All measured parameters remained within normal conditions, indicating stable rumen fermentation conditions. Although the differences in methane production and bacterial populations between treatments were not statistically significant, treatment P4 tended to show decreased methane production. These findings suggest that the combination of corn stover and red Calliandra up to a ratio of 60:40 is feasible to maintain the balance of rumen fermentation, with the potential to improve fermentation efficiency and reduce methane production. Further in vivo studies are needed to confirm these results under practical feeding conditions.

Keyword: Red Calliandra, Rumen pH, Microbial Populations, methane gas, in vitro

INTRODUCTION

The ruminant livestock sector plays a crucial role in providing animal protein and supporting national food security. Increasing ruminant livestock productivity is strongly influenced by the quality and availability of feed. The availability of forage in Indonesia often fluctuates due to seasonal changes and limited land. In addition to affecting livestock productivity, feed also plays a role in environmental impacts, particularly through the production of methane (CH_4) gas from the rumen fermentation process. Methane is a greenhouse gas that contributes to global warming and reflects the loss of feed energy that is not utilized by livestock. One widely developed alternative is the use of agricultural waste as an environmentally friendly feed source. Corn stover are the entire corn plant, including stalks, leaves, and harvested corn. Many livestock farmers in Indonesia use corn stover as livestock feed. Corn stover have a high fiber content and are available year-round, but their crude protein content is relatively low, so their use needs to be combined with feed ingredients containing higher nutrients to increase rumen fermentation efficiency (Hernaman et al., 2017).

To address these nutrient deficiencies, supplementation with protein-rich feed sources such as red calliandra *(Calliandra calothyrsus)* is necessary. Red calliandra *(Calliandra calothyrsus)* is a leguminous tree forage that can be used as an alternative livestock feed and is known as a protein-rich feed source. Red calliandra contains 20.84% crude protein (Susilawati and Khairani, 2017). However, calliandra contains anti-nutritional substances in the form of tannins, which can affect the fermentation process in the rumen.

The rumen fermentation process is significantly influenced by the pH of the rumen fluid. A favorable pH for rumen fermentation is between 6.0 and 7.0, allowing rumen microbial populations, such as bacteria and protozoa, to function optimally in degrading feed (Hernandez et al., 2020). If the pH drops below this range or rises, microbial activity decreases, inhibiting the fermentation process and reducing digestibility.

The bacterial and protozoan populations in the rumen are closely related to the fermentation process. Bacteria play a key role in fiber decomposition and volatile fatty acid (VFA) production, while protozoa play a role in maintaining microbial population stability and utilizing starch and protein from feed ingredients (Yanti et al., 2021). Changes in the feed ingredient balance can affect the number of these microbial populations.

Methane gas (CH_4) is a gas produced by methanogenic bacteria during the rumen fermentation process. Attention to methane (CH_4) production is crucial because it is a byproduct of rumen fermentation and contributes to greenhouse gas emissions. Ruminant livestock contribute significantly to greenhouse gas emissions Livestock, especially ruminants, produce methane gas CH_4 of 14.5% (Gerber, P. J., et al. 2013). Therefore, efforts to reduce methane production through feed formulations, such as the use of ingredients containing natural tannins, include red calliandra.

MATERIALS AND METHODS

Silage Production

Silage is made from a mixture of corn stover and red calliandra. The material is then chopped into small pieces using a machete. then mixed and added molasses that has been diluted with air in a ratio of 1:1.The finished silage is placed in airtight plastic jars and fermented for 22 days under anaerobic conditions. After fermentation, the silage is dried and ground into a fine powder using Hammer mill.

T1 = Silage with a ratio of 90% corn stover and 10% red calliandra.

T2 = Silage with a ratio of 80% corn stover and 20% red calliandra.

T3 = Silage with a ratio of 70% corn stover and 30% red calliandra.

T4 = Silage with a ratio of 60% corn stover and 40% red calliandra.

In vitro process

The in vitro process used the method of Theodorou et al. (1994). In vitro was performed by placing a 0.5 g sample into a pre-prepared vial, then adding 40 mL of McDougall's solution (artificial saliva) and 10 mL of dairy cow rumen. CO_2 gas was then circulated into the fermenter to create an anaerobic environment. The vial was sealed with a rubber seal and crimped, then stored in a water bath at 39-40°C. The vial was incubated for 24 hours, shaking and gas sampling every 2 hours. After 24 hours of incubation, the contents of the vial were collected and the total number of bacteria and protozoa measured under a microscope. Rumen fluid was collected from the Ciroyom Slaughterhouse from two cattle located at Jl. Arjuna No. 45, Husen Sastranegara District, Cicendo District, Bandung City, West Java Province.

Total Bacteria Measurement

The bacterial measurement process used the method of Tanuwiria et al. (2025). 20 µL of rumen fluid was mixed with 6980 µL of Hayem's solution. The mixture was diluted to 10-4 and then pipetted into the center of the counting chamber, which had previously been covered with a cover glass. Observations and counts were then performed under a microscope at 1000x magnification. The total bacterial count was performed in a small box within the counting chamber. The total bacterial count in 1 mL of rumen fluid is:

$$\text{Total of bacteria} = \frac{\left(\left(\frac{Avvrage\ bacteri}{5}\right) x\ \ 350\ x\ 1000\right)}{0{,}004}$$

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XIII Issue V May 2026

Total Protozoa Measurement

The bacterial measurement process used the method of Tanuwiria et al. (2025). 4.5 mL of formalin solution was mixed with 0.5 mL of rumen fluid. This fluid was allowed to stand for 30 minutes to allow the nuclei to stain. Afterward, observations were made under a microscope at 400x magnification. The number of protozoa in 1 mL of rumen fluid was calculated as follows:

number of protozoa= $Average\ of\ protozoa\ x\ \frac{1000}{100}\ x\ 7$

pH Measurement

The pH value of rumen fluid was measured using a pH meter that had been standardized with a buffer solution at pH 7 for ±10 minutes, then measured in a buffer solution at pH 4 for ±10 minutes. The cathode part was dipped into the solution for ±10 minutes until the number in the pH meter did not move, then the pH value was recorded (Hernaman et al., 2015)

CH4 Methane Gas Measurement

Methane gas was measured using gas chromatography. Gas was collected every two hours during the 24-hour incubation period using a 10 mL syringe. The resulting gas was collected in a 10 mL vial, which had been previously vacuumed. Total gas production over 24 hours was obtained by summing the volume of gas collected at each measurement interval. A 5 mL sample of gas was then transferred to a 5 mL Vacutainer tube for further analysis. Methane gas measurements were carried out using the gas chromatography method with the injector temperature set at 90°C and the column temperature at 75°C.

Data Analysis

This study employed an experimental method using a Completely Randomized Design (CRD) consisting of four treatments with five replications. The treatments were as follows:

T1 = Silage with a ratio of 90% corn stover and 10% red calliandra

T2 = Silage with a ratio of 80% corn stover and 20% red calliandra

T3 = Silage with a ratio of 70% corn stover and 30% red calliandra

T4 = Silage with a ratio of 60% corn stover and 40% red calliandra

The data obtained were then analyzed using analysis of variance (ANOVA), and significant differences between treatments were determined. Further evaluation was carried out using Duncan's multiple range test to identify differences between each treatment.

RESULT AND DISCUSSION

Table 1. The Effect of Corn Stover and Red Calliandra Ratio in Silage on Rumen pH, Bacterial Populations, Protozoa Populations and Methane Gas (In Vitro)

Parameter	Treatment			
	T1	T2	T3	T4
pH	6.868±0.0277^{ab}	6.836±0.030^{a}	6.862±0.029^{ab}	6.888±0.008^{b}
Protozoa (10^5 Cell/mL)	5.332±0.0798^{a}	5.082±0.468^{a}	4.56±0.073^{b}	4.23±0.135^{b}
Bacteria (10^9 Cell/mL)	3.352±0.5982^{a}	3.17±0.1568^{a}	3.022±0.139^{a}	3.166±0.081^{a}
Methane Gas(mM)	6.141±1.0439^{a}	5.55±0.5720^{a}	5.774±0.753^{a}	5.662±0.872^{a}

Based on ANOVA, only pH and protozoa population were significantly affected by treatment ($P \leq 0.05$), while bacterial population and methane production did not show significant differences among treatments ($P > 0.05$). Different superscript letters indicate significant differences.

pH

Observations showed that the ratio of corn stover and red calliandra significantly affected rumen pH ($P<0.05$). This difference indicates that variations in feed composition can influence rumen pH. The average rumen pH across all treatments ranged from 6.868 to 6.888. This pH value is within normal limits, indicating that rumen fermentation is proceeding smoothly.

Rumen pH is an indicator of the success of rumen fermentation. A good rumen pH, within the normal range of 6 to 7, is crucial for optimal rumen microbial fermentation. When the pH is within this range, rumen microorganisms can grow and degrade feed effectively, and microbial activity remains high, an indicator of good fermentation. A less-than-ideal pH will disrupt microbial activity and slow down the fermentation and feed degradation processes in the rumen of ruminants (Maulana et al., 2016).

Rumen pH is a crucial factor influencing rumen digestion (Perez et al., 2024). Maintaining rumen pH is crucial. A stable rumen pH allows rumen microbes to grow and develop well, producing volatile fatty acids (VFAs) and NH_3 Suharti et al., (2018). Likewise, as Faniyi (2024) stated, optimal rumen pH typically ranges between 6.0 and 7.0, supporting a diverse population of fiber-degrading bacteria essential for effective digestion.

Protozoa

Observations of protozoa populations showed significant affected ($P<0.05$). The average protozoa population ranged from 4.23 to 5.332 x 10^5 Cells/mL for each treatment. This protozoa population is still within the normal range for rumen protozoa, which averages around 10^5 to 10^6 cells/mL (Yasin et al., 2021). The results of the study also showed a downward trend in protozoa populations for each treatment.

Previous research has shown a similar finding. (Ningrat et al., 2019) found that adding lamtoro legumes reduced protozoa populations. Aban (2016) also found that using lamtoro legumes at higher doses reduced protozoa populations. This study supports the findings of previous research showing that the combination of corn stover and red calliandra legumes can affect protozoa populations but still under optimal conditions.

The decrease in protozoa numbers in this study was likely due to the tannin content in red calliandra. Tannins have antimicrobial properties that can inhibit the activity and growth of certain microbes in the rumen, including protozoa, by binding to proteins and interfering with microbial enzyme activity. This binding alters membrane permeability, disrupting membrane function and ultimately causing protozoan cell damage or lysis. Tannins can also bind microbial proteins and enzymes, disrupting protozoan metabolic activity. When enzymes are unable to function properly, protozoa cannot digest nutrients optimally and their growth is inhibited (Besharati, 2022).

Bacteria

The results of the study showed no significant effect on the bacterial population. Bacterial populations in each treatment ranged from 3,022 to 3,352 × 10^9 Cell/mL, indicating that rumen fermentation conditions were relatively uniform and stable. This indicates that the balance of corn stover and red calliandra in the given treatments provided sufficient fermentation substrate to support bacterial growth. According to (McDonald et al., 2010), the total bacterial population for rumen fermentation activity is 10^9 to 10^{11}. The bacterial population observed in this study was within the optimal range to support nutrient degradation and volatile fatty acid (VFA) production in the rumen.

The results showed relatively comparable bacterial populations across treatments. The increasing proportion of red calliandra in each treatment tended to stabilize the bacterial population. This stability was attributed to a supportive environment and sufficient nutrient intake for bacterial growth (Kusumaningrum et al., 2018). Research conducted by (Hidayat et al., 2020) aligns with research that suggests that combining energy-based feed with red calliandra legumes does not always reduce rumen microbial populations. This is because energy availability maintains fermentation activity and microbial growth, and the presence of rumen bacteria that can adapt to tannins keeps populations stable.

Methane Gas

Analysis of variance (Analysis of variance) of the ratio of corn stover and red calliandra did not significantly affect methane gas production (P>0.05), but there was a tendency to decrease. Methane concentrations ranged from 5.55 to 6.141 mM across all treatments. Methane production from each treatment showed a narrow range.

Methane gas in the rumen is naturally formed from CO^2 and H^2, catalyzed by methanogenic microbes as part of the feed fermentation process in the rumen (Yuliana, 2019). Methane gas production results from the interaction of protozoa and rumen bacteria. In the initial stage, carbohydrates from feed are fermented into volatile fatty acids (VFAs), which are used by livestock as an energy source, producing hydrogen gas (H_2) and carbon dioxide (CO^2) as byproducts. Methanogenic microorganisms (Archaea) then utilize the H_2 and CO_2 through methanogenesis, a process that involves the reduction of CO^2 by H_2, producing methane gas (CH_4). Previous research (Ridwan et al., 2014) found that increasing the use of silage containing red calliandra in in vitro rumen fermentation reduced methane concentration and total gas production by approximately 11–25%.

CONCLUSION

Based on the results of this research, it can be concluded that the combination of corn stover and red calliandra did not significantly impact bacterial populations and methane production, although there was a downward trend. This indicates that the combination of corn stover and red calliandra is still able to maintain stable rumen fermentation conditions, allowing bacterial activity and the fermentation process to proceed normally. However, the balanced feed treatment significantly affected rumen pH and protozoa populations, indicating that feed composition can influence the dynamics of certain microorganisms in the rumen ecosystem.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial support provided by the Academic Leadership Grant (ALG) under Grant Number (3208/UN6.J/PT.00/2025). This support was crucial in enabling the successful completion of this research project.

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